

cta Haematologica

International Journal of Haematology
Journal International d'Hématologie
Internationale Zeitschrift für Hämatologie

Official Organ of the European Division of the International Society of Haematology
General Secretary: J. W. STEWART, London

Founded 1948 by E. MEULINOWITZ, K. ROSEN and G. ROSENOW
Continued by H. LÖNN (1960-1977)

U. BUCHER, Bern	R. S. HILLMAN, Seattle, Wash.	
H. BRAUVESTEDEN, Lund	E. OSMUND, Seattle, Wash.	Y. NABHAN, Paris
J. H. DAVIS, Glasgow	E. A. LOEWEN, Leiden	L. SANCHEZ-MEDAL, Mexico City
A. J. EMBLEY, Philadelphia, Pa.	A. MARMONT, Genova	S. SAND, Frankfurt a. M.
J. FORSTER, Winnipeg	H. R. MARTI, Astu	
F. GARDNER, Galveston, Tex.	J. MCARTHUR, Seattle, Wash.	



Index Vol. 60, 1978

No. 1

Original Papers Originalarbeiten Travaux originaux

- EL-HADAD, M. A. F. and LEVYMAN, H. Interaction between Iron Deficiency and α -Thalassemia: the *in vitro* Effect of Haemin on α -Chain Synthesis 1
- RICCI, P., BACCARANI, M., ZACCARIA, A., SANTUCCI, M. A., and TURA, S. Clinical Contribution to the Knowledge of Hemopoietic Dysplasias: Long-Term Follow-Up of 13 Patients with Refractory Anemia 19
- GIOVANNONE, E., PADICIPATO, G. B., AMMONIO, M. V., GRABU, G., and DELL'AGATA, M. Erythrocyte Acetylcholinesterase Activity Changes in the Early Stages Anemic and Hypobaric Hypoxia or after CoCl_2 Treatment in Rabbits 21
- BARR, R. D. and WITT, J. Preliminary Evidence for the Common Origin of Lympho-Myeloid Complex in Man 29
- PERDUM, G. D. and EVANS, C. A. Significance of Mouse Red Cell Rosette-Forming Lymphocytes in Chronic Lymphocytic Leukemia 36
- MAYER, M., SPERLING, H., SCHARFER, J. and QUINER, W. Megakaryocyte Proliferation in May-Hegglin Anomaly 45

Short Communication Kurze Mitteilung Communication brève

- CHATTER, D., PLOTZKY, P. and SAVIN, H. Platelet Aggregation in Diabetic Retinopathy 53

Correspondence Briefe Lettres

- PAOLINO, W., LEVY, A., CARAMIELLO, L., and PAOLINO, F. Chronic Myeloid Leukemia Initiating as Acute Lymphoid Leukemia 56
- WANG, S. and KAM, Y. Intrathecal Methotrexate Causing Paraplegia in Middle-Aged Woman 59

Book Reviews Buchbesprechungen Livres nouveaux

62

No. 2

Original Papers Originalarbeiten Travaux originaux	
MILDER, M. S., COOK, J. D., and FINCH, C. A. Influence of Food Iron Absorption on the Plasma Iron Level in Idiopathic Hemochromatosis	65
ARCAJOY, A., ÇAYDAR, A. O. and BARACAN, E. Decreased Iron and Zinc Absorption in Turkish Children with Iron Deficiency and Geophagia	76
HYPAKMEZ, G., SÖZER, N., SÜLOGLU, G., and DÖNMEZ, S. Platelet Counts in Children with Iron Deficiency Anemia	85
MARMONT, A. M. Congenital Hypoplastic Anemia Refractory to Corticosteroids but Responding to Cyclophosphamide and Antilymphocytic Globulin. Report of a Case Having Responded with a Transitory Wave of Dyserythropoiesis	90
GIARDINO, O., MURCIA, F., MARTINO, F., MANNARINO, O., CORRADO, G., and MAGGIORI, G. Serum Lipid Pattern in β -Thalassemia	100
ROSE, R. A., MULDER, N. H., and NIEWEN, H. O. 'Classic and Acute Myelofibrosis. A Retrospective Study	108
BENJAMIN, D., DOUER, D., PICK, A. I., ZER, M., DINTIMAN, M., and PINCHAS, J. Peripheral Cryoglobulinemic Neuropathy in a Patient with Gaucher's Disease	117
Short Communication Kurze Mitteilung Communication brève	
DROZDZCZ, W., MOZSWEK, B., WRONKA, J., PORADZINSKA, H., JACZEWSKI, R., and BOGACKA-ZATORIKA, H. Megakaryocytic Leukemia. A Case of Unusual Outcome	122
Books Reviews Buchbesprechungen Livres nouveaux	126
Varia	128

No. 3

Original Papers Originalarbeiten Travaux originaux	
MORELL, A., MAURER, W., SEVARIL, F., and BARANDUN, S. Differentiation Between Benign and Malignant Monoclonal Gammopathies by Discriminant Analysis on Serum and Bone Marrow Parameters	129
MORELL, A., SEVARIL, F., HUSER, H. J., SPENGLER, G., and BARANDUN, S. Cytoplasmic Immunoglobulins in Bone Marrow Cells of Polyclonal and of Monoclonal Origin	137
BARTAL, A., MEKORI, T., HAASZ, R., COHEN, Y., and ROBINSON, E. Multiple Lymphocyte Transformation Tests by Phytohemagglutinin in Healthy Individuals. Transient Episodes of Decreased Lymphocyte Blastogenesis	148
BARTAL, A., BENTWICH, Z., MANNY, N., and IZAK, G. Ethical and Clinical Aspects of Chronic Lymphocytic Leukemia in Israel. A Survey on 288 Patients	161
KOVÁCS, P., BRUCH, C., HERBST, E. W., and FLEISCHER, T. M. Collection of <i>In vitro</i> Colony-Forming Units from Dogs by Repeated Continuous Flow Leukaphereses	177
CELADA, A., RUDOLF, H., HERREROS, V., and DONATH, A. Inorganic Iron Absorption in Subjects with Iron Deficiency Anemia, Achylia gastrica and Alcoholic Cirrhosis Using a Whole-Body Counter	18

No. 4

Original Papers · Originalarbeiten · Travaux originaux

- SCORLEO, G. MUSUMECI, S. PIZZARELLI, G. DI GREGORIO, L. FISCHER, A., and RUSO, G. β -Thalassemia in Sicily. Hematological and Biosynthetic Studies 191
- ODA, S., ODA, E., and TANAKA, K. R. Relationship of Density Distribution and Pyruvate Kinase Electrophoretic Pattern of Erythrocytes in Sickle Cell Disease and Other Disorders 201
- VERHOEF, N. J., KOTTERAGIN, M. J., MULDER, H. J. M., NOORDHOFS, P. J., and LEIJDE, R. Functional Heterogeneity of Transferrin-Bound Iron. Iron Uptake by Cell Suspensions from Bone Marrow and Liver and by Cell Cultures of Fibroblasts and Lymphoblasts 210
- REMOUSSI, L., DOLZY, C., PALESTRO, G., and PISTOLE, C. Paraproteinoemic Variety of Pure Cell Aplasia. Immunological Studies in 1 Patient 227
- GRATWOHL, A. A., BULL, M. I., GRAY, R. G. JR., NORTON, L., and KNUTSEN, T. Methotrexate and Citrovorum Factor after Histo-incompatible Allogeneic Bone Marrow Transplants in Dogs 233
- JORGENSEN, R. H. and MANSFIELD, A. O. A New Method for the Detection of the Plasminogen Activator Content of Vein Walls 243
- Case Report · Fallstudie · Pat. clinique
- CAVALLI, F., SONVIGO, R. W., ZUCKERMAN, A., DEUTSCHER, K., and RYNDZ, H. J. Non-Hodgkin Lymphoma Terminating in Acute Myelogenous Leukemia 250

No. 5

Original Papers · Originalarbeiten · Travaux originaux

- MATTHEW, Y., BHAGAMAT, J., and POLLACK, A. Prognostic Factors in Multiple Myeloma. A Retrospective Study Using Conventional Statistical Methods and Computer Program 257
- VIRELLA, G., LOPEZ-VIRELLA, M. F., LEVINE, J., OGAWA, M., and GONZALEZ, J. 'Idiopathic' Bence-Jones Proteinuria 269
- BACICALUPO, A., SANTOZ, G., PIAGGIO, G., RUSSO, M., LINT, M. T. AN, CARILLA, M., DAMASO, E. E., RAFFO, M. C., and MARMONT, A. M. Lymphoid Antigens on Blast Cells in the Agranular Metamorphosis of Chronic Myelogenous Leukemia 280
- CAMMAGNI, G. and PADUA, S. Defect of Bone Marrow Granulocyte Reserve in Liver Cirrhosis Evaluated with Ethocholanolone 291
- REITZ, F. P., HETTEL, A. DO P., COSTA, M., REIDEN, O. R. AN, and OLSON, R. Comparison between the Unsaturated Plasma Folate Binder and a new Labeled Plasma Folate Binder 296
- STOLTZ, J. F. and NICOLAS, A. Study of Amino Groups of the Human Platelet Membrane 304
- FRIEDM, K., OLSON, L.-B., VILM, L., and KUTTI, J. Peripheral Platelet Count in Response to Salbutamol Before and After Adrenergic Beta-Receptor Blockade 310

Case Report Fallstudie Falt clinique

- VORONOFF D. A. BENJAMIN, J. KAPLAN, H. and DVLANSKY A. Chronic Granulocytic Leukemia, Neutrophilic Type, with Paraproteinemia (IgA Type K) 316

No. 6

Original Papers Originalarbeiten Travaux originaux

- BEAVER, G. H. and GRATZER, W. B. Binding of Protoporphyrin and Haemin to Human Spectrin 321
- JOHNSON, R. and VUORIO, P. Studies on Red Cell Flexibility in Spherocytosis Using a Polycarbonate Membrane Filtration Method 329
- CORROCHER, R. PACHON M. L. BAMBARA, L. M. AMEROSETTI, A., and DE SANDRI, G. Folic Acid Binding Protein in Chronic Granulocytic Leukemia The Effect of Methotrexate 341
- SANDLER, S. G. SCHILLER, G. RUSSO A. MURUSIEL, S. and RACHAGLEWITZ, E. A. Blood Group Phenotypes and the Origin of Sickle Cell Hemoglobins in Sicilians 350
- CAOCCI, L. ALBERTI, M. BURLAI, P., and CORDA, R. Screening Coagulation Tests and Clotting Factors in Homozygous β -Thalassemia 358
- Book Reviews Buchbesprechungen Livres nouveaux 363
- Index rerum 370
- Index autorum 384

Interaction between Iron Deficiency and α -Thalassaemia the *in vitro* Effect of Haemin on α -Chain Synthesis

M. A. F. EL HAZMI and H. LEHMANN

University Department of Clinical Biochemistry Addenbrooks's Hospital, Cambridge

Key Words. Saudi Arabia α -Thalassaemia Iron deficiency Haemin
Biosynthesis

Abstract. Two conditions are liable to lower the α/β globin biosynthesis ratio in reticulocytes: iron deficiency and α -thalassaemia. The present paper studies the effect of haemin on reticulocytes from 12 patients who have α -thalassaemia and/or are iron deficient. The α/β globin biosynthesis ratio was improved in all these cases. 4 showed initially an α/β synthesis ratio usually associated with α -thalassaemia type-1 on the addition of haemin the ratio rose to that associated with α -thalassaemia type-2. In the other 8 patients the ratio was initially typical for α -thalassaemia type-2, and on addition of haemin the ratio became normal. It is suggested that in iron deficient patients a diagnosis of α -thalassaemia type-1 or type-2 cannot be made unless haemin has been added to the test system. If this is not done iron deficiency alone can cause the α/β globin synthesis ratio to resemble that associated with α -thalassaemia type 2, and iron deficiency in combination with α -thalassaemia type-2 can cause the ratio to resemble that typical for α -thalassaemia type-1.

Reticulocytes from 8 α -thalassaemic patients without iron deficiency did not show marked haemin effect (<5%), and in 1 patient with iron overload, the ratio actually fell by about 10%.

Introduction

Both iron deficiency and thalassaemia, one affecting the haem, and the other the globin, are well known to cause by themselves hypochromic anaemia. Their interaction is therefore of interest.

Iron starvation [1-7] iron chelators [8] sideroblastic anaemia [5, 6, 9, 10] and lead poisoning [4, 6, 9] cause secondary reduction of globin

biosynthesis, and conversely inhibition by cycloheximide of globin biosynthesis in reticulocytes reduces haem synthesis. However there have been few reports concerning the effects of the combination of thalassaemia and iron deficiency on globin biosynthesis [11-12]. Taken together these reports suggest a preferential reduction of α -chain biosynthesis in iron deficiency.

On describing a new haemoglobin Riyadh [12] the carrier of the new variant was iron deficient and had α -thalassaemia. The α/β globin biosynthesis ratio was 0.67 ± 0.04 which was in the range of α -thalassaemia type-1 (2 α -chain genes of 4 deleted). On adding haemin the α/β synthesis ratio rose to within a range associated with α -thalassaemia type-2 (1 α -chain gene of 4 deleted).

The aim of the present work was to study the interaction *in vitro* between iron deficiency and α -thalassaemia. After initial experiments in which iron had shown no effect on biosynthesis of globin [13] haemin was chosen as the stimulant.

Materials and Methods

10 non-anaemic and 74 anaemic patients, aged between 6 months and 50 years were investigated. All were Saudi Arabs admitted to the Central Hospital of Riyadh.

Blood films for red cell morphology were usually prepared from a finger prick. Haematological investigations, were carried out on heparinised blood as soon after venipuncture as possible using standard methods [14]. Iron [15] and protein [16] concentrations were determined subsequently using a Technicon autoanalyser II. Haemolysates were prepared and electrophoresis was carried out on agarose film in 0.05 M sodium barbital buffer [17]. Hb A₂ was determined by elution after cellulose acetate electrophoresis [18] and Hb F by alkali denaturation [19].

For *in vitro* biosynthesis, haemin solutions were prepared according to the method of Hunt *et al* [20] and reticulocyte-enriched blood was prepared as described previously [12]. The incorporation of ³H leucine was allowed to proceed in the absence (control) and the presence of haemin (33 mM) for 60 min at 37 °C after which it was terminated by adding ice-cold saline containing 1 mM cycloheximide. The cells were then washed three times with cold saline and lysed by repeated freezing and thawing. Globin was prepared from haemolysates of cells incubated with and without haemin and analyzed at the same time. Whole cell globin was dissolved in initial chromatography buffer [21] and reduced under N₂ at room temperature for 2 h. Thereafter the protein solution was gel filtered on a 0.9 × 15 cm column of Sephadex G-25 (coarse) equilibrated with the same buffer. The protein eluate was fractionated on a CM 23 cellulose column [21]. The eluate was monitored at 280 nm and 10-ml fractions were collected at 60 ml/h. The radioactivity incorporated into the globin chains was determined [22] in 1 ml from each fraction in a Tru

Table 1 Hematological, biochemical findings and haemoglobin synthesis results of normal subjects (controls)

Case	RBC 10 ⁶ /l	Hb g/dl	PCV l/l	MCV fl	MCH pg	MCHC g/dl	HbA ₂ %	HbF %	Total iron concentration μ mol/l	TIBC μ mol/l	Total protein concentration g/l	Globin chain ratios radio- specific activity (non- α) (β) cpm/chain cpm/200 nm
3N	4.50	14.5	0.42	93	32.2	34.52	2.1	1.5	16	54	75	1.08 0.96
2N	4.15	14.0	0.42	101	33.7	33.33	2.4	0.9	14	59	76	1.02 0.95
5N	4.10	12.5	0.40	97	30.5	31.25	2.7	2.0	16	62	65	0.98 1.01
2	4.30	12.5	0.39	90	29.0	32.03	3.4	0.7	15	62	69	0.93 0.96
1	3.90	11.5	0.36	97	29.5	30.26	3.5	0.8	20	56	57	1.04 0.99
4	4.00	13.5	0.52	105	33.7	32.14	2.2	0.8	17	54	71	0.94 0.97
411	3.77	12.1	0.38	100	32.0	31.84	3.6	1.2	14	59	69	0.96 1.02
6	4.99	16.5	0.48	96	32.7	33.96	2.7	0.8	16	59	75	1.05 1.03
7N	4.70	14.7	0.44	94	31.3	33.40	3.5	1.5	22	56	76	0.98 0.95
436	4.56	14.5	0.44	100	33.2	32.95	3.4	0.8	25	53	70	0.91 0.96
Normal ranges												
Male	3.5 \pm 1	13.5 \pm 2.5	0.47 \pm 0.03 0.07 \pm	83 \pm 8	29.5 \pm 2.5	33 \pm 2	2.5 - 3.5	<1.0	14-22	54-74	63-83	Mean 0.98 SD \pm 0.07 0.98 \pm 0.03
Female	4.8 \pm 1	14 \pm 2.5	0.42 \pm 0.05						10-28			

Table II Haematological findings, iron and protein concentrations and haemoglobin composition in iron deficient subjects (group A)

Case	Age/sex	Hb g/dl	PCV l/l	Total concentration		Haemoglobin constitution			
				iron $\mu\text{mol/l}$	protein g/l	HbA ₂ %	HbF %	HbA %	HbS %
7	35 Y/F	9.5	0.28	5	57	2.6	2.0	95.4	-
8C	33 Y/F	8.0	0.27	5	60	2.5	1.4	95.1	-
M11	19 Y/F	9.5	0.30	4	63	2.7	1.5	95.8	-
12C	35 Y/F	8.0	0.24	4	70	2.1	1.1	96.8	-
11C	2 Y/M	7.0	0.22	3	70	1.1	1.7	97.2	-
14	14 Y/F	11.7	0.35	8	72	2.5	1.5	95.0	-
5C	42 Y/M	11.7	0.36	7	68	2.4	0.7	96.9	-
10	23 Y/F	6.5	0.19	5	67	2.2	0.9	96.9	-
6A	6 Y/M	9.0	0.27	4	67	2.4	1.2	96.4	-
3	43 Y/M	5.7	0.17	4	62	2.7	1.2	67.1	28.0
5	52 Y/M	8.0	0.24	6	58	2.9	1.5	65.6	30.0
10A	6 M/F	9.5	0.31	4	69	1.9	33.1	40.0	25.0

cerlab Corn Matie 200' and the optical density was estimated using a Zeiss recording spectrophotometer (DMR 21). The radioactivity was expressed as total counts per chain fractions or as specific activity expressed as cpm per fraction at 280 nm.

Results

The individual values for $\alpha\beta$ globin synthesis ratio for non-anaemic controls (fig 1 table I) were taken to represent the normal range for adults.

In 12 iron-deficient patients, group A (table II) the incorporation of ^3H leucine into globin chains revealed a reduced $\alpha\beta$ globin synthesis ratio (fig 1). Haemin produced not only an overall increase in the rate of globin synthesis, but also raised the $\alpha\beta$ globin synthesis ratio.

There were two sub-groups in this category. One showed $\alpha\beta$ synthesis ratios of 0.60 ± 0.06 and the other ratios of 0.82 ± 0.04 . On incubation in media with added haemin, the $\alpha\beta$ ratio in the first was raised but was not brought up to normal while in the latter the globin chain ratios approached unity.

Whilst there was, in group A, a definite response to haemin on $\alpha\beta$ globin synthesis, no such response was found in 9 patients (group B).

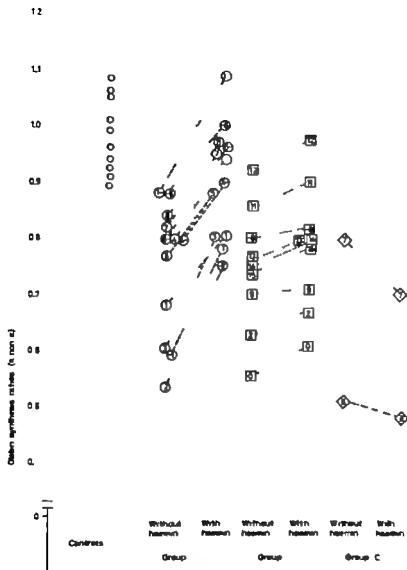


Fig. 1 The effect of haem on α -globin synthesis. Group A: iron-deficient patients. Group B: anemic patients without iron deficiency. Group C: one patient with iron overload (top) and one with protein malnutrition (bottom).

Table III Haematological findings, iron and protein concentrations and haemoglobin composition in anaemic patients without iron deficiency (group B)

Case	Age/sex	Hb g/dl	PCV l/l	Total concentration		Haemoglobin consti- tution		
				iron $\mu\text{mol/l}$	protein g/l	HbA ₁ %	HbF %	HbA %
8	11 Y/M	7.1	0.20	26	72	2.5	2.2	95.3
9	11 Y/F	8.6	0.26	16	63	2.1	1.0	96.9
11	51 Y/M	8.6	0.26	23	42	2.6	1.3	95.9
12	35 Y/M	8.3	0.24	14	57	1.3	1.2	97.5
15	16 Y/F	8.6	0.26	31	70	2.8	0.6	96.6
14	14 Y/F	8.0	0.24	14	68	2.3	0.9	96.8
8A	4 Y/M	12.1	0.37	14	71	1.2	0.8	98.0
2	6 Y/M	6.8	0.21	13	73	2.3	1.1	96.6
1	15 Y/F	11.5	0.36	15	70	3.5	1.6	94.9

Table IV Haematological findings, iron and protein concentrations and haemoglobin composition in a patient with protein malnutrition (6C) and one with iron overload (12C) (group C)

Case	Age/sex	Hb g/dl	PCV l/l	Total concentration		Haemoglobin consti- tution		
				iron $\mu\text{mol/l}$	protein g/l	HbA ₁ %	HbF %	HbA %
6C	12 Y/M	3.7	0.12	4	6	1.9	2.6	95.5
12C	33 Y/F	6.3	0.19	70	65	2.5	1.1	96.4

whose anaemia was not associated with iron deficiency (table III). Another 2 subjects (group C, table IV) 1 with iron overload (17C) and 1 protein deficient (6C) showed a reduced α -chain synthesis (fig. 1 cases 7 and 8 respectively) when haemin was added.

Discussion

Iron deficiency by itself has been reported to lower the *in vitro* α non α chain biosynthesis ratio though the effect was marked only when the

globin was prepared from stroma-free haemolysate [7]. When the stroma was not removed, the ratio still fell from a control value of 1.03 to 0.88 ± 0.04 . This suggests that the iron-depleted α -chains attach themselves to the stroma.

Our results suggest that iron deficiency can interact with α -thalassaemia, causing a further reduction in the synthesis of α -globin chain. The combination of iron deficiency and α -thalassaemia type 2, each of which by itself produces similar blood pictures, may on combination cause an α -thalassaemia type 1-like condition.

This may be the explanation for the change on addition of haemin of an α/β globin synthesis ratio typical for α -thalassaemia type 1 (0.60 ± 0.06) to one typical for α -thalassaemia type-2 (0.78 ± 0.02) [23, 24]. The haemin effect thus discloses the genetical part of the α -chain deficiency and can alter the diagnosis of α -thalassaemia. The second type of patient with an initially thalassaemia type 2 range of synthesis returned to normal on the addition of haemin. Here haemin again corrected a possible diagnosis of α -thalassaemia. It is obvious that the diagnosis of α -thalassaemia by biosynthesis cannot be reliable in iron deficiency unless it includes a control in which haemin is added. This control can convert the diagnosis of 'silent' α -thalassaemia (type 2) to one of normality and one of moderately severe α -thalassaemia (type 1) to that of α -thalassaemia type 2. Both these α -thalassaemia types are essentially mild and their differentiation may have no great clinical implications. However it is of value to differentiate between microcytosis responsive to iron treatment and refractory microcytosis due to α -thalassaemia. In addition, it is often essential to make the diagnosis for genetic counselling, because homozygotes for type 1 will die from hydrops fetalis, and heterozygotes for types-1 and 2 will suffer from haemoglobin H disease.

Haem (or iron) has an effect on protein synthesis in general [25, 26] and indeed on the synthesis of globins other than the α -chain [27, 32, 33]. However the effect on the α -chain exceeds that on the other proteins and non- α globin chains.

Acknowledgements

This study was carried out during the tenure of Research Scholarship of M. A. F. E. H. from the University of Riyadh, Saudi Arabia. The University support for the project is gratefully acknowledged together with the help of Drs A.-R. SWALEH and M. SADERK and co-operation of Dr M. I. ASSORA in whose department the haematology was performed. H. L. received support from the Medical Research Council.

Table III Haematological findings, iron and protein concentrations and haemoglobin composition in anaemic patients without iron deficiency (group B)

Case	Age/sex	Hb g/dl	PCV l/l	Total concentration		Haemoglobin constitution		
				iron $\mu\text{mol/l}$	protein g/l	HbA ₂ %	HbF %	HbA %
8	11 Y/M	7.1	0.20	26	72	2.5	2.2	95.3
9	23 Y/F	8.6	0.26	16	63	2.1	1.0	96.9
11	51 Y/M	8.6	0.26	23	42	2.6	1.5	95.9
12	35 Y/M	8.3	0.24	14	57	1.3	1.2	97.5
15	16 Y/F	8.6	0.26	31	70	2.8	0.6	96.6
14	14 Y/F	8.0	0.24	14	68	2.3	0.9	96.8
8A	4 Y/M	12.1	0.37	14	71	1.2	0.8	98.0
~	6 Y/M	6.8	0.21	13	73	2.3	1.1	96.6
1	15 Y/F	11.5	0.36	15	70	3.5	1.6	94.9

Table IV Haematological findings, iron and protein concentrations and haemoglobin composition in a patient with protein malnutrition (6C) and one with iron overload (12C) (group C)

Case	Age/sex	Hb g/dl	PCV l/l	Total concentration		Haemoglobin constitution		
				iron $\mu\text{mol/l}$	protein g/l	HbA ₂ %	HbF %	HbA %
6C	12 Y/M	3.7	0.12	4	6	1.9	2.6	95.5
12C	33 Y/F	6.3	0.19	70	65	2.5	1.1	96.4

whose anaemia was not associated with iron deficiency (table III). Another 2 subjects (group C table IV) 1 with iron overload (17C) and 1 protein deficient (6C) showed a reduced α -chain synthesis (fig 1 cases 7 and -8 respectively) when haemin was added.

Discussion

Iron deficiency by itself has been reported to lower the *in vitro* α non α chain biosynthesis ratio though the effect was marked only when the

globin was prepared from stroma-free haemolysate [7]. When the stroma was not removed, the ratio still fell from a control value of 1.03 to 0.88 ± 0.04 . This suggests that the iron-depleted α -chains attach themselves to the stroma.

Our results suggest that iron deficiency can interact with α -thalassaemia, causing a further reduction in the synthesis of α -globin chain. The combination of iron deficiency and α -thalassaemia type-2, each of which by itself produces similar blood pictures, may on combination cause an α -thalassaemia type-1-like condition.

This may be the explanation for the change on addition of haemin of an $\alpha:\beta$ globin synthesis ratio typical for α -thalassaemia type 1 (0.60 ± 0.06) to one typical for α -thalassaemia type-2 (0.78 ± 0.02) [23, 24]. The haemin effect thus discloses the genetical part of the α -chain deficiency and can alter the diagnosis of α -thalassaemia. The second type of patient with an initially thalassaemia type-2 range of synthesis returned to normal on the addition of haemin. Here haemin again corrected a possible diagnosis of α -thalassaemia. It is obvious that the diagnosis of α -thalassaemia by biosynthesis cannot be reliable in iron deficiency unless it includes a control in which haemin is added. This control can convert the diagnosis of 'silent' α -thalassaemia (type-2) to one of normality and one of moderately severe α -thalassaemia (type-1) to that of α -thalassaemia type 2. Both these α -thalassaemia types are essentially mild and their differentiation may have no great clinical implications. However it is of value to differentiate between microcytosis responsive to iron treatment and refractory microcytosis due to α -thalassaemia. In addition, it is often essential to make the diagnosis for genetic counselling, because homozygotes for type-1 will die from hydrops fetalis, and heterozygotes for types-1 and 2 will suffer from haemoglobin H disease.

Haem (or iron) has an effect on protein synthesis in general [25, 26] and indeed on the synthesis of globins other than the α -chain [27, 32, 33]. However the effect on the α -chain exceeds that on the other proteins and non- α globin chains.

Acknowledgements

This study was carried out during the tenure of a Research Scholarship of M. A. P. E. H. from the University of Riyadh, Saudi Arabia. The University support for the project is gratefully acknowledged together with the help of Drs. A. R. SWALEH and M. SADOUK and co-operation of Dr. M. I. ANSARA in whose department the haematology was performed. H. L. received support from the Medical Research Council.

References

- 1 GRAYZEL, A. I. HÖSCHNER, P. and LONDON I. M. The stimulation of globin synthesis by heme. *Proc. natn. Acad. Sci. USA* 55 650-655 (1966)
- 2 HUNT R. T. HUNTER, A. R. and MUNRO, A. J.. Control of haemoglobin synthesis. A difference in the size of polysomes making α and β chains. *Nature, Lond* 220 481-483 (1968)
- 3 WAXMAN H. and RABINOVITZ, M.. Iron supplementation *in vitro* and the state of aggregation and function of reticulocyte ribosomes in hemoglobin synthesis. *Biochem. biophys. Res. Commun.* 19 538-545 (1965)
- 4 WAXMAN H. and RABINOVITZ, M. Control of reticulocyte polyribosome content and hemoglobin synthesis by heme. *Biochim. biophys. Acta* 129 369-379 (1966)
- 5 WHITE, J. M. and ALL, M. Globin synthesis in sideroblastic anemia. II. Effect of pyridoxine, δ -aminolevulinic acid and haem, *in vitro*. *Br J Haemat.* 24 481-489 (1973).
- 6 WHITE, J. M. and HOFFBRAND A. V.. Haem deficiency and chain synthesis. *Nature, Lond.* 248 88 (1974)
- 7 BEN BARAT L. MOZEL, M. and RAMOT B.. Globin synthesis in iron-deficiency anemia. *Blood* 44 551-555 (1974)
- 8 RABINOVITZ, M. and WAXMAN, H. Dependence of polyribosome structure in reticulocytes on iron. Implications on the tape theory of haemoglobin synthesis. *Nature, Lond.* 206 897-900 (1965).
- 9 WHITE, J. M. and HARVEY D. R. Defective synthesis of α and β globin chains in lead poisoning. *Nature, Lond* 236 71-73 (1972).
- 10 WHITE, J. M., BRAIN M. C., and ALL, M. Globin synthesis in sideroblastic anemia. I α and β peptide chain synthesis. *Br J Haemat.* 20 263-275 (1971).
- 11 WALFORD D. and DEACON, R.. The effect of iron deficiency on globin chain biosynthesis in normals and thalassaemics in 3rd Meet. Int. Soc. Haematology European and African Division, London 1975, Abstr 42, p. 18.
- 12 EL HAZMI, M. A. P. and LUDMANN, H. Hemoglobin Riyadh - $\alpha_2\beta_2$ (120(GH 3)Lys-Asn). A new variant found in association with α -thalassaemia and iron deficiency Hemoglobin 1 59-74 (1976-1977)
- 13 EL HAZMI, M. A. P. PhD thesis Cambridge (1977)
- 14 DACEY, J. V. and LEWIS, S. M. Practical haematology 5th ed (Churchill, London 1975).
- 15 STOEKEY L. L. Ferrozine. A new spectrophotometric reagent for iron. *Analyt. Chem.* 42 779-781 (1970).
- 16 WECHSLERBAUM, T. E. Accurate and rapid method for determination of proteins in small amounts of blood serum and plasma. *Am. J clin. Path* 10 40-49 (1946)
- 17 Corning Eel Agarose Film/Cassette Electrophoresis system. No.001 91 046 A ed. (1974)
- 18 MARENGO-ROWE, A. J. Rapid electrophoresis and quantitation of haemoglobins on cellulose acetate. *J clin. Path.* 18. 790-792 (1965).

- 19 BETTER, K., MARTZ, H. R., and SCHLICHT, L. Estimation of small percentages of foetal haemoglobin. *Nature, Lond.* 184 1877-1878 (1959).
- 20 HUNT, T., VANDERHOFF, G. A., and LONDON, I. M. Control of globin synthesis. The role of heme. *J. molec. Biol.* 66 471-481 (1972).
- 21 CLEGG, J. B., N. DOWTON, M. A., and WEATHERALL, D. J. Abnormal human haemoglobins. Separation and characterization of the α - and β -chains by chromatography and determination of two new variants: Hb Chesapeake and Hb J (Bangkok). *J. molec. Biol.* 19 111 108 (1966).
- 22 HUNT, T., HUNTER, T. and MURGO, A. Control of haemoglobin synthesis. Distribution of ribosomes on the messenger RNA for α - and β -chains. *J. molec. Biol.* 35 31-45 (1968).
- 23 KAN, N. W., SCHWARTZ, E., and NATHAN, H. G. Globin chain synthesis in the alpha thalassaemia syndromes. *J. clin. Invest.* 47 2515-2522 (1968).
- 24 FOOTRAKLY, S., SAPPALPA, S., WASE, H., NA-NAKORN, S. and SWANCK, R. H. Haemoglobin synthesis in 28 obligatory cases for α -thalassaemia trait. *Humangenetik* 29 121-126 (1975).
- 25 BRIZARD, Y., ROOVIEN, R., and LONDON, I. M. Effect of hemin on the synthesis of hemoglobin and other proteins in mammalian cells. *Proc. natn. Acad. Sci. USA* 70 1022-1026 (1973).
- 26 LODES, H. Biogenesis of reticulocyte membrane proteins by membrane free polyribosomes. *Proc. natn. Acad. Sci. USA* 70 1526-1530 (1973).
- 27 JOSEPHSON, A. M., MARRI, M. S., SMOER, L., DWORIN, D., and SCHER, K. Starch block electrophoretic studies of human hemoglobin solutions. II. Results in cord blood, thalassaemia and other hematologic disorders. Comparison with Threlks electrophoresis. *Blood* 13 543-551 (1958).
- 28 LEVINE, R. H., LICHTMAN, H. C. and LEVINE, J. Effect of iron-deficiency anaemia on the metabolism of the heterogenic haemoglobins in sickle cell trait. *Nature, Lond.* 202 499-501 (1964).
- 29 WASE, P., DISTASAKOCHIAN, P. and NA-NAKORN, S. The effect of iron deficiency on the levels of Hb A_2 and E. *J. Lab. clin. Med.* 71 85-91 (1968).
- 30 KUCZYNSKI, A. The relationship between the serum iron concentration and erythrocytic haemoglobin A_2 level. *J. Med.* 2 136-142 (1971).
- 31 STEDER, J., MARTZ, H. R., and DRAKE, D. Decreased haemoglobin A_2 concentration in iron deficiency anemia. *Acta haemat.* 45 77-81 (1971).
- 32 LEHMANN, H. and HOFSTEENGE, R. G. Men's haemoglobins; 1st ed, p. 176 (North-Holland, Amsterdam, 1966).
- 33 O'BARR, R. T. The effect of iron deficiency on the expression of Hb H. *Blood* 41 853-856 (1973).

Prof. H. LEHMANN, University Department of Clinical Biochemistry Addenbrooke's Hospital, Cambridge CB2 2QR (England)

Present address: Department of Biochemistry Tennis Court Road, Cambridge CB2 1QP (England)

Clinical Contribution to the Knowledge of Hemopoietic Dysplasias Long-Term Follow-Up of 13 Patients with Refractory Anemia¹

PAOLO RICCI, MICHELE BACCARANI ALFONSO ZACCARIA
MARIA ALESSANDRA SANTUCCI and SANTE TURA

Chair of Hematology University of Bologna, and Division of Hematology
St. Orsola's University Hospital, Bologna

Key Words Hemopoietic dysplasias Preleukemia Dyserythropoiesis
Refractory anemia Chromosomes

Abstract Refractory anemia (RA) is an hematologic disorder at risk of developing into a malignancy (hemopoietic dysplasia). Information on hemopoietic dysplasias is useful for sharpening the appropriate diagnostic criteria and for the search of an appropriate therapy. Moreover hemopoietic dysplasias provide an interesting opportunity in humans, for studying a developing leukemia, or a disease situated at the boundary of leukemia. This paper reports on the evolution of RA in 13 patients followed up more than 2 years, through clinical observation, blood and marrow examination, and karyotype analysis. 4 of these 13 patients developed acute or subacute myeloid leukemia. An additional patient died because of severe thrombocytopenia. In these 5 patients, dyserythropoiesis was accompanied by thrombocytopenia, marrow myeloblastosis, and nonrandom chromosome abnormalities.

In humans, there is a broad and as yet undefined spectrum of hematologic disorders which share some characters of acute myeloid leukemia (AML) namely abnormal proliferation and maturation of myeloid precursors, leading to insufficient production of erythrocytes, granulocytes, and thrombocytes [1 5 11 12, 20 24]. It has been proposed recently to

Work supported in part by Centro Bolognese per lo Studio delle Leucemie e dei Linfomi.

group these disorders under the general heading of hemopoietic dysplasias, i.e. conditions at risk of developing into a malignancy [1-7]. The distinction between hemopoietic dysplasias and AML is not sharp. This is due to the objective difficulty of separating leukemic from nonleukemic conditions [1-10] and to the relative paucity of adequate information on the clinical characters, laboratory features, and evolution of the multifarious hemopoietic dysplasias [12, 22, 23]. The interest in these disorders lies both in the necessity for sharper diagnostic criteria, and the shortage of adequate therapy [4-10, 22]. Moreover hemopoietic dysplasias provide a natural model, in humans, for investigation of a developing leukemia, or of a disease situated at the boundary of leukemia [1-10, 12, 22]. The importance of these points was emphasized during a symposium recently held in Paris, leading to a proposition for an international registry for hemopoietic dysplasias [8].

Amongst hemopoietic dysplasias, much space is occupied by a chronic anemia of the elderly already recognized at the beginning of the century [13] and described today under the general heading of refractory anemia (RA) [4-5].

In a previous paper [22] we provided an extensive description of RA, based on the study of 16 patients, and on the review of another 304 cases, together with data on cytology, cytochemistry, cytogenetics, cell kinetics, iron kinetics, and the growth patterns of marrow cells in soft agar. We report here on the evolution of the disease in 13 patients with a follow-up longer than 2 years. All these patients have been continuously under our care, making it possible to correlate their clinical course with appropriate laboratory investigation, including marrow and karyotype studies. Information on this disorder is somewhat confusing, because of the heterogeneity of the cases reported under the same or similar headings. Accordingly this paper has been written in a way hopefully as to allow the reader to identify each patient, by providing the basic clinical and hematological data, and to trace the way of the disease after diagnosis.

Patients and Methods

13 patients with RA have been first admitted between October 1971 and December 1974, over a period of 39 months.

A marrow differential was performed at diagnosis in all patients, by counting at least 1,000 consecutive nucleated cells. A second marrow specimen was obtained for

Table I Blood and Iron kinetic data at diagnosis

Patients	Hb g/dl	Reticulo- cytes $\times 10^3/\mu\text{l}$	Platelets $\times 10^3/\mu\text{l}$	PMN(N)/ μl	Serum iron $\mu\text{g/dl}$	Plasma iron turnover mg/h/l	Hb synthesis g/day/l	Red cell ^{59}Fe incorporation %
1 GA	6.1	1.8	94	1,944	290	1.53	5.5	18
2 TM	9.5	68.0	80	2,378	80	1.48	6.7	37
3 CN	5.7	30.6	65	1,222	150	1.56	6.0	20
4 ZG	5.0	2.8	270	2,040	175	2.29	7.7	14
5 ZL	5.6	28.2	46	1,666	260	1.1	4.4	5
6 SA	8.3	24.0	198	1,610	295	ND	ND	ND
7 MB	10.4	74.8	211	1,674	225	1.58	6.2	14
8 PR	4.9	64.0	294	2,808	145	4.33	18.0	20
9 GG	6.3	3.7	247	2,304	200	2.40	6.5	10
10 GeG	5.6	93.3	213	2,176	215	ND	ND	ND
11 CC	11.0	111.3	67	1,320	215	ND	ND	ND
12 MG	4.3	6.5	218	1,426	165	ND	ND	ND
13 MA	10.4	29.0	185	920	205	1.49	6.3	18
Normal range	12.0- 18.0	25.0- 75.0	150- 400	2,000- 6,000	80- 150	0.2- 0.40	1.0- 1.6	80- 100

Hb = Hemoglobin, PMN(N) = polymorphonuclears (neutrophilic), ND = not done.

a differential count shortly before death, or at the time of writing, in all but 4 patients. In 5 patients, marrow differentials were performed serially during the course of the disease. Myeloblasts were separated from promyelocytes and myelocytes according to the absence and respectively the presence of cytoplasmatic granules, as described elsewhere [22]. Erythroblasts were separated into four cytological compartments ($E_1 = E_p$, E_2 , and E_3) according to the classification proposed by KILLMANN [9].

The karyotype of marrow cells was studied at diagnosis in all patients, by analyzing under the microscope 13-22 well-spread metaphases, prepared according to the direct method described by TRO and WILANO [21]. Chromosomes were identified according to the Denver classification. Karyotype analysis was repeated in all but 5 patients shortly before death, or at the time of writing. In 3 patients, the karyotype was analyzed serially during the course of the disease.

Iron kinetic studies were performed at diagnosis in all but 5 patients according to the method of POLLYCOVE and MORTIMER [16]. Pearl's staining was performed as described by DAVIS and LEWIS [2].

Normal marrow differential was determined by counting a total of 14,707 consecutive nucleated marrow cells obtained from 6 healthy volunteers.

Table II Marrow data at diagnosis

Patient	Cellularity	% of all nucleated marrow cells							G/E ratio	Ring sideroblast
		MB	PMC + MC(N)	M/MC + PM/N(N)	E ₁₋₄	E ₅	E ₆	E ₇		
1 OA	+++~	11.4	8.7	13.1	2.1	7.7	39.6	12.4	0.5	+
2 TM	++++	20.5	15.0	8.0	1.2	3.1	30.2	10.3	1.0	-
3 CN	++++	3.6	14.0	5.6	7.2	9.0	37.4	14.4	0.3	-
4 ZO	++++	2.4	12.4	17.1	7.4	21.4	29.0	5.2	0.5	+
5 ZL	+++++	2.6	26.6	47.4	2.2	4.1	9.9	5.5	3.5	+
6 SA	++++	1.3	10.3	15.8	2.0	3.8	45.6	14.8	0.4	+
7 MB	++++	1.0	12.1	20.1	1.5	6.4	43.2	8.5	0.6	+
8 FR	+++++	0.9	6.3	17.2	10.0	14.1	33.0	10.8	0.3	+
9 GG	++++	0.6	5.9	19.7	4.1	10.4	35.3	16.4	0.4	+
10 GG	+++±	3.1	13.0	26.7	7.2	■	20.1	11.0	0.9	+
11 CC	+++~	1.7	16.9	27.3	5.1	■	20.9	8.6	1.0	+
12 MG	++++	1.3	9.3	24.8	10.5	15.2	23.1	■	0.6	+
13 MA	+++±	1.6	13.8	27.9	0.7	3.8	30.2	12.9	0.9	+
normal	+ ± - -	1.1	15.6	41.9	0.6	1.6	10.7	14.2	2.2	-
		± 0.5	± 2.4	± 7.5	± 0.3	± 0.6	± 3.7	± 3.2		

MB = Myeloblasts, PMC + MC(N) = promyelocytes and myelocytes (neutrophilic), M/MC + PM(N) = metamyelocytes and polymorphonuclears (neutrophilic), E₁₋₄ = proerythroblasts, ■ = basophilic erythroblasts, E₆ = polychromatic erythroblasts, E₅ = orthochromatic erythroblasts, G/E = granulo/erythroid.

Mean of 6 normal marrow ± 1 SD

Results

Features of the Disease at Diagnosis

Table I shows blood counts and iron kinetic data at diagnosis. The absolute monocyte count (not reported in the table) was normal, and no patient had either blast cells or erythroblasts in the peripheral blood.

Marrow differentials at diagnosis are shown in table II. The bone marrow cellularity was higher than normal in all patients. The granulo/erythroid (G/E) ratio was much lower than normal in all cases but patient 5. A net excess of myeloblasts was recorded in 2 patients only. The majority of the erythroblasts were basophilic and polychromatic. After Pearl's staining, many erythroblasts appeared as ring sideroblasts in 8/13 but 2 cases.

Table III The karyotype at diagnosis

Patients	Number of metaphases				Annotations
	hypo-diploid	normal	pseudo-diploid	hyper-diploid	
1 GA	8/22	12/22	1/22	1/22	5 E ₁₆ - metaphases
2 TM	9/20	0/20	9/20	4/20	18 metaphases with 2 subtelocentric markers
3 CN	19/21	0/21	2/21	0/21	20 C - metaphases
4 ZG	0/19	3/19	4/19	12/19	9 47 C + metaphases
5 ZL	10/19	6/19	2/19	1/19	nonspecific aneuploidy
6 SA	9/20	9/20	1/20	1/20	nonspecific aneuploidy
7 MB	12/20	4/20	4/20	0/20	nonspecific aneuploidy
8 PR	9/19	4/19	6/19	0/19	nonspecific aneuploidy
9 GG	9/19	3/19	4/19	3/19	6 C + metaphases
10 GeG	11/22	8/22	2/22	1/22	12 E ₁₆ - metaphases
11 CC	2/17	15/17	0/17	0/17	normal
12 MG	9/26	13/26	1/26	3/26	nonspecific aneuploidy
13 MA	4/13	8/13	0/13	1/13	nonspecific aneuploidy

The karyotype profile at diagnosis is reported in table III. All but 1 patient had a highly abnormal karyotype, with a prevalence of hypodiploid metaphases. Only patient 4 had many hyperdiploid cells. Chromosome gain and loss were apparently random in many cases (nonspecific aneuploidy) but in some patients a variable proportion of the metaphases analyzed had missed or gained an E₁₆ or a C chromosome. All cells of patient 2 carried two subtelocentric markers.

Evolution of the Disease

The course of the disease is summarized in table IV. Patients 1 and 2 developed AML. Patient 3 died because of a massive bleeding from the gastrointestinal tract, associated with severe thrombocytopenia and granulocytopenia. Patients 4 and 5 developed subacute myeloid leukemia and died. Patients 6-9 died 26-53 months after diagnosis, and patients 10-13 are alive, 26-43 months after diagnosis, without any evidence of leukemic evolution or marrow failure.

Details on the evolution of the disease in patients 1-5 are provided in table V. Thrombocytopenia developed in the only patient (no 4) who had a normal platelet count at diagnosis. Marrow myeloblastosis increased or

Table IV Clinical evolution

Patients sex, age	Prediagnostic phase		Postdiagnostic phase		Cause of death
	duration months	symptoms	duration months	symptoms and complications	
1 OA M, 72 years	4	anemia	6	anemia, infections, hepato- splenomegaly AML after 5 months	AML
2 TM F 75 years	2	anemia, infections	8	anemia, infections, bleed- ing AML after 6 months	AML
3 CN F 59 years	11	anemia, purpura	16	anemia, recurrent purpura	massive gastrointes- tinal bleeding
4 ZG F 69 years	24	anemia	26	anemia, hepatomegaly con- gestive heart failure sub- acute myeloid leukemia after 11 months	subacute myeloid leukemia
5 ZL M, 67 years	2	anemia, infections	27	anemia, infections, sub- acute myeloid leukemia after 13 months	subacute myeloid leukemia
6 EA F 76 years	15	anemia	26	anemia, hepatomegaly diabetes mellitus	portal hypertension, marrow gastrointes- tinal bleeding
7 MB M, 68 years	7	anemia	35	anemia, congestive heart failure, hepatitis	congestive heart failure, hepatitis
8 PR M, 63 years	24	anemia	44	anemia, hepatospleno- megaly diabetes mellitus	congestive heart failure
9 GG M 64 years	36	anemia	53	anemia, hepatomegaly diabetes mellitus	congestive heart failure
10 OcG M, 67 years	27	anemia	76 +	anemia	(alive)
11 CC F 72 years	18	anemia, purpura	30 +	anemia, mild recurrent purpura	(alive)
12 MG F 50 years	43	anemia	36 +	anemia, hepatitis, splenomegaly	(alive)
13 MA F 64 years	11	anemia, cholelithiasis	43 +	anemia	(alive)

Age at diagnosis.

Interval from first laboratory evidence of anemia to diagnosis of RA.

+ Indicates the patient is still alive.

Table V Blood, marrow and karyotype changes in patients 1-5

Patient and clinical course	Time of study	Platelets $\times 10^3/\mu\text{l}$	PMN $\times 10^3/\mu\text{l}$	Marrow MB %	Karyotype
1 GA dead at 6 months with AML	diagnosis	94	1.9	11.4	aneuploid, 5/22 E14 - cells
	5 months	35	1.8	81.0	ND
	diagnosis	80	0.4	20.5	aneuploid, 18/20 cells with 2 subtelocentric markers
	2 months	44	0.4	18.8	ND
	6 months	57	0.5	26.5	ND
2 TM dead at 8 months with AML	7 months	16	0.1	51.2	aneuploid, with the same markers as at diagnosis
	diagnosis	65	1.2	3.6	aneuploid, 20/21 C- cells
	8 months	40	0.9	ND	ND
	15 months	2	0.5	ND	ND
3 CN dead at 16 months, with thrombocytopenia and severe gastrointestinal bleeding	diagnosis	270	2.0	2.4	aneuploid, 9/19 C+ cells
	4 months	253	1.8	0.9	ND
	15 months	278	1.4	17.8	aneuploid, 13/21 C+ cells
	26 months	41	3.1	ND	ND
4 ZL dead at 27 months with subacute myeloid leukemia	diagnosis	46	1.7	2.6	nonspecific aneuploidy
	7 months	58	2.2	1.3	ND
	13 months	47	1.9	43.2	ND
	18 months	27	2.4	57.6	aneuploid, 7/20 C+ cells
	24 months	59	1.4	45.8	aneuploid, 9/20 C+ cells

PMN = Polymorphonuclear neutrophils, MB = myeloblasts, ND = not done.

developed in all cases. The karyotype profile remained abnormal or became abnormal in all cases.

Patients 6-9 died without evidence of leukemic evolution or marrow failure. Their blood and marrow picture remained unchanged all through the course of the disease. Their karyotype, which was aneuploid at diagnosis, with random chromosome gain and loss (nonspecific aneuploidy) did not undergo any measurable change.

Patients 10-13 are alive and well at the time of writing, 25-42 months after diagnosis. Their blood and marrow picture, as well as their karyotype, have remained constant.

Much knowledge of RA is scattered and dispersed, notwithstanding its frequency and the studies by DREYFUS *et al* [5] either for the identification of the patients studied, or for the clinical and hematological evolution. The consequence of the intermediate and complex nature of the disorder. At the Paris Symposium [8] it was emphasized that the list of laboratory investigation useful for better knowledge of RA is endless. In practice, only a minor proportion of such studies can be performed effectively at a single Institution, and the suspected multicentric cooperation [8] which is necessary to accomplish such a formidable task, is not easy to realize. The severity of the disease and the old age of most patients add further difficulty. In effect, even the more sophisticated studies are of a limited value if they are not repeated during the course of the disease, and the course itself is not carefully recorded.

The minimum common denominator of the patients reported in this paper was anemia (table IV) due to decreased red cell production because of ineffective erythropoiesis. Ineffective erythropoiesis was documented by iron kinetic studies (table I), absolute erythroid hyperplasia, and relative prevalence of early erythroblasts in the marrows (table II).

It is suggested that recognition of such a prominent defect of erythropoiesis should be considered, at least for the time being, as essential for separating RA from other hemopoietic dysplasias or preleukemic states.

The second common feature of these patients was provided by chromosome studies (table III). Though the relevance of the karyotype to the diagnosis of RA is still unsettled, the data suggest that a normal karyotype is rare in this disorder. These data are in agreement with a number of previous cytogenetic studies [3, 14, 15, 19]. It should not be overlooked that in 7 patients chromosome abnormalities were apparently random (nonspecific aneuploidy), while in 6 patients the observation was more consistent with nonrandom changes. Recent investigation [18] suggests that the application of more refined techniques, such as the banding method (which was not available at our Institution at the time most patients were studied) will possibly identify a higher proportion of nonrandom abnormalities, and will allow to establish whether there are chromosome changes specific of the disease, or markers.

Dyserythropoiesis and chromosome abnormalities were accompanied

Table V7 Relationship between the course of the disease and laboratory data

	Patients 1-5	Patients 6-13
Granulocytopenia (PMN $< 2,000/\mu\text{l}$)	3/5	5/8
Thrombocytopenia ($< 100,000/\mu\text{l}$)	4/5	1/8
Marrow M1B $> 10^6$	7/5	0/8
Nonrandom chromosome abnormalities	5/5	1/8
Survival from first laboratory evidence of anemia	10-50 months	41-89 months
Survival from diagnosis of RA	6-27 months	46-53 months

Patients 1-5 died because of leukemia or thrombocytopenia. Patients 6-13 died because of nonhematological complications (4 patients), or are still alive without any evidence of leukemia (4 patients)

in many cases by mild to moderate thrombocytopenia and/or granulocytopenia (table I) and in 2 cases also by an excess of marrow myeloblasts. Analysis of previous reports [22] indicates that in this disorder the frequency of granulocytopenia approaches 50% of the cases, that of thrombocytopenia 20% and that approximately 1 out of 10 patients has an excess of myeloblasts in the marrow

The disease remained stable without changes of blood and marrow picture, in 8 patients (table IV). Patients 6-9 died because of complications secondary to chronic anemia and frequent blood transfusions, 26-53 months after diagnosis, and 41-89 months after the first laboratory evidence of anemia. Whether these 8 patients would have developed leukemia, would they have survived or followed up longer remains a matter of speculation

The course of the disease was quite different in patients 1-5 (table V), 4 of whom died with leukemia. It could be argued that patients 1 and 2 had AML from the very beginning. This is a distinct possibility which depends on what criteria are fixed for the diagnosis of AML. As previously outlined such criteria are not sharp. Also it should not be overlooked that many patients with marrow myeloblastosis do not progress towards overt leukemia for years [4]. Patients 4 and 5 developed marrow myeloblastosis followed by leukemia later after diagnosis. Their leukemia was defined as subacute for its duration (without cytotoxic therapy) the low number of blasts or abnormal mononuclear cells circulating in the blood,

and the lack of severe granulocytic dysplasia, with an hyperdiploid karyotype, with an hyperdiploid

Hemopoietic dysplasias develop into a malignancy [of developing leukemia was apparent] when dyserythropoiesis, or nonrandom chromosome changes, or both, as also suggested by previous reports [3, 4, 6, 12, 14, 17]. These criteria are obviously rough and provisional, but it is probable that in hemopoietic dysplasias characterized by dyserythropoiesis and anemia, the distance from leukemia can be roughly measured by the degree of the involvement of the other myeloid cell lines, and by the specificity of karyotype changes.

Patients had an abnormal

conditions at risk of developing (table VI) the risk when anemia and dyserythropoiesis, marrow myeloblastosis, as also suggested by previous reports [3, 4, 6, 12, 14, 17]. These criteria are obviously

rough and provisional, but it is probable that in hemopoietic dysplasias characterized by dyserythropoiesis and anemia, the distance from leukemia can be roughly measured by the degree of the involvement of the other myeloid cell lines, and by the specificity of karyotype changes.

References

1. BERNARD, J. Preleukemic states. *Blood Cells* 2: 5-7 (1976).
2. DACEY, J. V. and LEWIS, S. M. *Practical haematology* 3rd ed., pp. 84-86 (Churchill, London 1963).
3. DE GROUCHY, J., DE NIVA, C., ZITTOUK, R. et BOUSSIER, J. Analyses chromosomiques dans l'anémie sidéroblastique idiopathique acquise. *Nouv. Revue fr. Hémat.* 6: 367-388 (1966).
4. DREYFUS, B. Preleukemic states. *Blood Cells* 2: 33-35 (1976).
5. DREYFUS, B., ROCHANT, H. et SOLIAT, C. Anémies éfractaires enzymopathies acquises des cellules-souches hématopoïétiques. *Nouv. Revue fr. Hémat.* 9: 65-86 (1969).
6. EASTMAN, P. M., SCHWARTZ, R. and SCHERER, S. L. Distinctions between idiopathic ineffective erythropoiesis and De Guelmo's disease: clinical and biochemical differences. *Blood* 40: 487-499 (1972).
7. INSERM Symposium on Hemopoietic Dysplasias (Preleukemic States): General discussion. *Blood Cells* 2: 347-351 (1976).
8. INSERM Symposium on Hemopoietic Dysplasias (Preleukemic States): General discussion. *Blood Cells* 2: 357-359 (1976).
9. KILLMANN, S. A. Cell classification and kinetic aspects of normoblastic and megakaryoblastic erythropoiesis. *Cell Tissue Res.* 3: 217-228 (1970).
10. KILLMANN, S. A. Preleukemia: does it exist? *Blood Cells* 2: 81-105 (1976).
11. KUMDER, J. P., LEE, G. R., WINTHROP, M. H. and CARTWRIGHT, G. E. Idiopathic refractory sideroblastic anemia. Clinical and laboratory investigation of 17 patients and review of the literature. *Medicine Baltimore* 50: 139-159 (1971).
12. LIDMAN, J. W. and BAIRD, G. C. Jr. The preleukemic syndrome. Clinical and laboratory features, natural course, and management. *Blood Cells* 2: 11-31 (1976).

Table 11 Relationship between the course of the disease and laboratory data

	Patients 1-5	Patients 6-13
Granulocytopenia (PMN < 4,000/ μ l)	3/5	5/8
Thrombocytopenia (< 100,000/ μ l)	4/5	1/8
Marrow MB > 10%	2/5	0/8
Nonrandom chromosome abnormalities	5/5	1/8
Survival from first laboratory evidence of anemia	10-50 months	41-89 months
Survival from diagnosis of RA	6-27 months	76-53 months

Patients 1-5 died because of leukemia or thrombocytopenia. Patients 6-13 died because of nonhematological complications (4 patients), or are still alive without any evidence of leukemia (4 patients).

in many cases by mild to moderate thrombocytopenia and/or granulocytopenia (table I) and in 2 cases also by an excess of marrow myeloblasts. Analysis of previous reports [22] indicates that in this disorder the frequency of granulocytopenia approaches 50% of the cases, that of thrombocytopenia 20%, and that approximately 1 out of 10 patients has an excess of myeloblasts in the marrow.

The disease remained stable without changes of blood and marrow picture, in 8 patients (table IV). Patients 6-9 died because of complications secondary to chronic anemia and frequent blood transfusions, 26-53 months after diagnosis and 41-89 months after the first laboratory evidence of anemia. Whether these 8 patients would have developed leukemia, would they have survived or followed up longer remains a matter of speculation.

The course of the disease was quite different in patients 1-5 (table V) 4 of whom died with leukemia. It could be argued that patients 1 and 2 had AML from the very beginning. This is a distinct possibility which depends on what criteria are fixed for the diagnosis of AML. As previously outlined, such criteria are not sharp. Also it should not be overlooked that many patients with marrow myeloblastosis do not progress towards overt leukemia for years [4]. Patients 4 and 5 developed marrow myeloblastosis followed by leukemia later after diagnosis. Their leukemia was defined as subacute for its duration (without cytotoxic therapy) the low number of blasts or abnormal mononuclear cells circulating in the blood

Erythrocyte Acetylcholinesterase Activity Changes in the Early Stages of Anemic Hypobaric Hypoxia or after CoCl_2 Treatment in Rabbits

ELVIO GIOVANNINI, GIOVANNI B. PRENCIPATO, M. VITTORIA AMBROSINI,
GIULIOLA GRASSI and MASSIMO DELL'AGATA

Institute of General Biology Faculty of Medicine and Surgery
Chair of General Physiology Faculty of Sciences, University of Perugia, Perugia,
and II Chair of General Biology and Zoology Faculty of Medicine and Surgery
University of Chieti, Chieti

Key Words. Acid base balance Anemic hypoxia CoCl_2 treatment Erythrocyte AChE Hypobaric hypoxia

Abstract. The authors studied the changes of erythrocyte acetylcholinesterase (AChE) activity in rabbits respectively subjected to bleeding, hypobaric hypoxia and CoCl_2 treatment, in such conditions rise of the enzyme activity was observed. Similar increases take place also as a consequence of intravenous infusions carried out by using lactic or hydrochloric acid. The authors suggest that these increases in the erythrocyte AChE activity are connected with the decrease of the pCO_2 that occurs in the various experimental conditions achieved.

Although the function of erythrocyte acetylcholinesterase (AChE) has been the object of various experimental studies, it is not yet well known. An examination of the literature relative to this subject shows that various factors that are able to alter the erythrocyte membrane and reduce its osmotic resistance, also produce *in vitro* a decrease of activity in the enzyme studied [7]. Furthermore, this activity is reduced in the presence of various hemolytic diseases [2, 5-6] the mechanisms that lead to such enzymatic activity alterations are still unknown. It is also known that AChE, like to other erythrocyte enzymes, shows a higher activity in the younger red cells and in their precursors [3] such an activity afterwards

- 13 LUZZATTO, A. M.. Sull'anemia grave megaloblastica senza reperto ematologico corrispondente (anemia pseudo-aplastica). *Rivista Veneta di Scienze Mediche* 47 193-198 (1907).
- 14 NOWELL, P. C. Marrow chromosome studies in 'preleukemia' further correlation with clinical course. *Cancer* 28. 513-518 (1971)
- 15 PIERRE, R. V.. Cytogenetic studies in preleukemia. studies before and after transition to acute leukemia in 17 subjects. *Blood Cells* 1 163-170 (1975).
- 16 POLLYCOVE, M. and MORTIMER, L. The quantitative determination of iron kinetics and hemoglobin synthesis in human subjects. *J. clin. Invest.* 40 753-764 (1961).
- 17 REIZENTEN, P., LAGERLÖF B., SKÄRBERG, K. O., CARLMARK, B., KOCK, Y. and TORER, S. Alterations in erythropoiesis preceding leukemia. *Acta haemat.* 54 152-158 (1975)
- 18 ROWLEY J. D. BLAIRDELL, R. K., and JACOBSON, L. O. Chromosome studies in preleukemia. I Aneuploidy of group C chromosomes in three patients. *Blood* 27 782-799 (1966)
- 19 ROWLEY J. D.. Editorial review: the role of cytogenetics in hematology. *Blood* 48 1-7 (1976).
- 20 SAADET, M. I. and LIDMAN J. W. Preleukemia. The hematologic syndrome preceding acute leukemia. *Am. J. Med.* 55 38-48 (1973)
- 21 TIPO, J. H. and WHANG, J. Chromosome preparations of bone marrow cells without prior *in vitro* culture or *in vivo* colchicine administration. *Stain Technol.* 37 17-20 (1962)
- 22 TURA, S., BACCARANI, M., RICCI, P., ZACCARIA, A. e MÜLLER BERAT N. Anemia disritropoietica idiopatica acquisita. *Atti 24 Congr. Soc. Ital. Emat.*, p. 65-144 (1973).
- 23 ZITTOUN R., CERTIN, M., AUDEBERT A. et BOUSSER, J. Evolution des anémies réfractaires. Rapport avec les leucémies myélo-monocytaires. *Ann. Méd. Interne*, 125 593-601 (1974)
- 24 ZITTOUN R., DIEBOLD J., BERNADOU, A., DAO, C., ZITTOUN, J., CADOU M., PROST-DYONAKOVIC, J., BILSKY PASQUIER, G. et BOUSSER, J. Diagnostic des états préleucémiques et des leucémies myélo-monocytaires et atypiques. *Nouv. Revue fr. Hémat.* 13 380-390 (1973).

Erythrocyte Acetylcholinesterase Activity Changes In the Early Stages of Anemic and Hypobaric Hypoxia or after CoCl_2 Treatment in Rabbits

ELVIO GIOVANNINI, GIOVANNI B. PRINCIPATO, M. VITTORIA AMBROSINI,
GIULIO GRASSI and MASSIMO DELL'AGATA

Institut of General Biology Faculty of Medicine and Surgery
Chair of General Physiology Faculty of Sciences, University of Perugia, Perugia,
and II Chair of General Biology and Zoology Faculty of Medicine and Surgery
University of Chieti, Chieti

Key Words. Acid base balance Anemic hypoxia CoCl_2 treatment Erythrocyte AChE Hypobaric hypoxia

Abstract The authors studied the changes of erythrocyte acetylcholinesterase (AChE) activity in rabbits respectively subjected to bleeding, hypobaric hypoxia and CoCl_2 treatment; in such conditions rise of the enzyme activity was observed. Similar increases take place also as consequence of intravenous infusions carried out by using lactic or hydrochloric acid. The authors suggest that these increases in the erythrocyte AChE activity are connected with the decrease of the pCO_2 that occurs in the various experimental conditions achieved.

Although the function of erythrocyte acetylcholinesterase (AChE) has been the object of various experimental studies, it is not yet well known. An examination of the literature relative to this subject shows that various factors that are able to alter the erythrocyte membrane and reduce its osmotic resistance also produce *in vitro* a decrease of activity in the enzyme studied [7]. Furthermore, this activity is reduced in the presence of various hemolytic diseases [2, 5, 6] the mechanisms that lead to such enzymatic activity alterations are still unknown. It is also known that AChE, like to other erythrocyte enzymes, shows a higher activity in the younger red cells and in their precursors [3] such an activity afterwards

decreases with the ageing of the erythrocytes, but it is not known whether these activity changes may be due to a progressive enzyme loss or to reduced catalytic efficiency [7]. In addition very little is known about the existence of treatments or conditions that are able to stimulate *in vivo* the erythrocyte AChE activity.

In the course of preliminary experiments concerning some erythrocyte enzymes of rabbits, we detected an AChE activity increase during the first hours after an acute bleeding. We therefore thought it as well to study the activity of the enzyme considered more thoroughly by extending the observations to other experimental conditions that were able to produce like the bleeding, states of hypoxia with erythropoietic stimulation [8] for this purpose we considered the hypobaric hypoxia and CoCl_2 treatment which causes an intracellular hypoxia [9]. Our recent studies showed that the above-mentioned experimental conditions produce a rise in blood lactic acid level and acid base balance changes [1]. We wished therefore to evaluate the activity of the enzyme studied also in conditions of metabolic acidosis experimentally achieved.

Material and Methods

The research was carried out by using rabbits of both sexes, approximately 6 months old and weighing 3 kg, fed with commercial rabbit pellets and tap water *ad libitum*, the various experiments were performed by using 5 groups of 6 animals to be subjected respectively to bleeding (I), hypobaric hypoxia (II), CoCl_2 treatment (III), lactic acid (IV) or HCl (V) infusion. Bleedings were carried out by taking off 70 ml of blood/kg of body weight by intracardiac puncture. hypobaric hypoxia was performed in a decompression chamber at 0.42 atm for 12 h. CoCl_2 treatment was carried out by giving a single intramuscular injection of a 0.25 M $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ solution in bidistilled water at the dose level of 250 mg/mol/kg of body weight. The lactic acid or HCl infusions were performed by phlebotomy in the ear marginal vein and using 0.1 M solutions at the flow of 0.8 and 0.3 ml/min, respectively: such treatments were stopped after 3 h because more prolonged infusions gave sometimes rise to hemolytic phenomena.

Arterial blood (1–1.5 ml) was sampled anaerobically into heparinized plastic syringes by intracardiac puncture from the animals belonging to the various groups such a sampling was performed in groups I, II and III at 0, 0.75, 1.5, 3, 4.5, 5, 6, and 12 h, and in groups IV and V at 0, 1.5, and 3 h. In all cases erythrocyte AChE activity and hemoglobinemia were evaluated. regarding groups IV and V blood pH and pCO_2 were also measured. In addition, reticulocyte levels were evaluated in each group of rabbits before the beginning and at the end of the various experiments.

Table 1 Erythrocyte AChE activity (IU/g Hb) in blood samples taken at successive time intervals from bled (A), kept in hypobaric chamber (B) or CoCl_2 -treated (C) rabbits

Time, h	Mean \pm SE	Difference from initial	Individual totals	Variance analysis source of variation	sum of squares	d.f.	mean squares	F
A								
Initial	10.8 \pm 0.689		93.8	times	59.68	6	9.95	19.5*
0.75	12.1 \pm 0.752	1.3	97.2	indiv	87.61	5	17.52	34.36*
1.5	13.2 \pm 1.169	2.4	102.9	error	15.39	30	0.51	
3	13.9 \pm 0.679	3.1**	98.8	total	162.68	41		
4.5	14.1 \pm 0.746	3.3**	90.3					
6	14.2 \pm 0.658	3.4**	71.6					
12	14.0 \pm 0.439	3.2**						
B								
Initial	10.3 \pm 0.598		90.5	times	36.20	6	6.03	14.03**
0.75	10.8 \pm 0.603	0.5	93.3	indiv	66.33	5	13.27	30.85**
1.5	11.7 \pm 0.822	1.4**	73.9	error	13.02	30	0.43	
3	11.8 \pm 0.662	1.5**	90.3	total	115.55	41		
4.5	12.2 \pm 0.576	1.9*	76.1					
6	12.9 \pm 0.481	2.6	72.1					
12	13.0 \pm 0.494	2.7*						
C								
Initial	9.7 \pm 0.998		78.7	times	120.63	6	20.11	7.67**
0.75	12.0 \pm 0.767	2.3	83.2	indiv	107.94	5	21.59	8.65*
1.5	12.2 \pm 0.750	2.5	84.4	error	78.57	30	2.62	
3	15.3 \pm 1.512	5.6	64.5	total	307.14	41		
4.5	10.6 \pm 0.897	0.9	75.2					
6	11.0 \pm 0.604	1.3	101.7					
12	10.5 \pm 0.781	0.8						

d.f. = Degrees of freedom F = variance ratio (*) significant (**) highly significant.

Mean values \pm SE and statistical assessment of the variability due to different variation sources by means of variance analysis

Comparison between the averages of the initial and subsequent values by means of Duncan test.

The AChE activity was determined according to the method of ELLMAN *et al* [4] at room temperature of 25 °C and expressed in IU (nmol of acetylthiocholine hydrolyzed/min)/g hemoglobin was measured as cyanoacetylmoglobin by evaluating spectrophotometrically the absorbance at 540 nm. The various above-mentioned de-

Table II Erythrocyte AChE activity¹ (IU/g Hb) pH¹ pCO₂¹ (mm Hg) in blood samples taken at successive time intervals from rabbits treated by intravenous infusion of 0.1 N lactic acid

Time, h	Mean ± SE	Difference from initial ²	Individual totals	Variance analysis source of variation	sum of squares	d.f.	mean squares	F
<i>AChE</i>								
Initial	9.9 ± 0.857		28.8 39.4	times	19.75	2	9.875	7.53
1.5	12.3 ± 0.927	2.4	44.2 31.0	indiv	63.80	5	12.760	9.80*
3	12.0 ± 0.982	2.1	32.8 29.4	error	13.02	10	1.30	
				total	96.57	17		
<i>pH</i>								
Initial	7.45 ± 0.010		22.40 2.39	times	0.0033		0.00165	< 1
1.5	7.48 ± 0.018	0.03	22.5 2.35	indiv	0.0210	5	0.00420	2.13
3	7.47 ± 0.030	0.02	22.60 2.40	error	0.0197	10	0.00197	
				total	0.0440	17		
<i>pCO</i>								
Initial	33.8 ± 0.986		85.9 81.4	times	19.45	2	9.725	23.2*
1.5	28.1 ± 1.461	5.7	101.2 94.5	indiv	128.49	5	25.698	620*
3	26.1 ± 1.599	7.7*	77.0 88.1	error	41.44	10	4.144	
				total	362.38	17		

Footnotes and explanations as in table I

terminations were performed by using a Beckman Dk 2A spectrophotometer. The blood pH and pCO₂ values were measured at 37 °C by using a Gas Analyzer Radiometer BMS3. The reticulocyte counts were carried out on the basis of ~2000 erythrocytes microscope samples were prepared with blood stained by brilliant cresyl blue.

Results

The results obtained in the course of the various experiments were subjected to statistical assessment by variance analysis. For each of the parameters studied the significance of the variations with time was evaluated. We considered variations caused by both the treatments and the individual variability which was almost always present in a significant degree. This method allowed the reduction of experimental error and was applicable because measurements in the same animals at successive time

Table III. Erythrocyte AChE activity (IU/g Hb), pH, $p\text{CO}_2$ (mm Hg) in blood samples taken at successive time intervals from rabbits treated by intravenous infusion of 0.1% HCl

Time, h	Mean \pm SE	Difference from initial	Individual totals	variance analysis source of variation	sum of squares	df	mean squares	F
AChE								
Initial	9.5 \pm 0.919		26.4 36.3	times	10.53	2	5.265	15.26
1.5	11.0 \pm 1.040	1.5	38.1 23.0	indiv	71.99	5	14.398	41.73**
3	11.1 \pm 0.766	1.6	28.2 37.9	error	3.45	10	0.345	
				total	85.97	17		
pH								
Initial	7.44 \pm 0.036		22.05 22.41	times	0.029	2	0.0145	8.53**
1.5	7.37 \pm 0.031	0.07*	22.27 21.91	indiv	0.050	5	0.0100	5.88*
3	7.35 \pm 0.033	0.09*	22.23 22.09	error	0.017	10	0.0017	
				total	0.096	17		
$p\text{CO}_2$								
Initial	31.9 \pm 0.944		91.9 90.8	times	142.87	2	71.435	11.1**
1.5	28.6 \pm 1.735	3.3	80.0 95.4	indiv	143.05	5	28.610	4.44**
3	25.0 \pm 1.735	6.9*	84.6 70.3	error	64.36	10	6.436	
				total	350.28	17		

Footnotes and explanations as in table I

Intervals were always carried out. Comparisons between initial and subsequent values were performed by Duncan's test. Results and statistical evaluations are shown in tables.

Erythrocyte AChE activity related to the hemoglobin concentration, significantly increases in the groups of animals subjected to various kinds of hypoxia (table I) such an increase is already present at 0.75 h in the bled or CoCl_2 -treated animals and at 1.5 h in the rabbits kept in a hypobaric chamber. The enzyme activity returns to normal values within 4.5 h in the CoCl_2 -treated animals, whereas in the other groups it still shows high values at 12 h.

Also the animals subjected to infusion with acids (table II-III) show – within the considered time limits – increases of the erythrocyte AChE activity that are detectable at 1.5 and 3 h moreover they show significant decreases of the $p\text{CO}_2$, whereas an acidotic state with significant decrease

Table II Erythrocyte AChE activity¹ (IU/g Hb) pH¹ pCO₂¹ (mm Hg) in blood samples taken at successive time intervals from rabbits treated by intravenous infusion of 0.1 N lactic acid

Time, h	Mean ± SE	Difference from Initial ²	Individual totals	Variance analysis source of variation	sum of squares	d.f.	mean squares	F
<i>AChE</i>								
Initial	9.9 ± 0.857		28.8 39.4	times	19.75	2	9.875	7.58
1.5	12.3 ± 0.927	2.4	44.2 31.0	Indiv	63.80	5	12.760	9.80 ³
3	12.0 ± 0.982	2.1	32.8 29.4	error	13.0	10	1.302	
				total	96.57	17		
<i>pH</i>								
Initial	7.45 ± 0.010		22.40 22.39	times	0.0033	2	0.00165	<1
1.5	7.48 ± 0.018	0.03	22.25 22.35	Indiv	0.0210	5	0.00420	1.13
3	7.47 ± 0.030	0.02	22.60 22.40	error	0.0197	10	0.00197	
				total	0.0440	17		
<i>pCO₂</i>								
Initial	33.8 ± 0.986		85.9 81.4	times	192.45		96.225	23.2
1.5	28.1 ± 1.461	5.7	101.2 94.5	Indiv	128.49	5	25.698	6.30 ³
3	26.1 ± 1.399	7.7	77.0 88.1	error	41.44	10	4.144	
				total	362.38	17		

Footnotes and explanations as in table I

terminations were performed by using a Beckman DK 2A spectrophotometer. The blood pH and pCO₂ values were measured at 37 °C by using a Gas Analyzer Radiometer BMS3. The reticulocyte counts were carried out on the basis of 1,000 erythrocytes; microscope samples were prepared with blood stained by brilliant cresyl blue.

Results

The results obtained in the course of the various experiments were subjected to statistical assessment by variance analysis. For each of the parameters studied the significance of the variations with time was evaluated. We considered variations caused by both the treatments and the individual variability, which was almost always present in a significant degree. This method allowed the reduction of experimental error and was applicable because measurements in the same animals at successive time

Table III Erythrocyte AChE activity (IU/g Hb), pH, $p\text{CO}_2$ (mm Hg) in blood samples taken at successive time intervals from rabbits treated by intravenous infusion of 0.1 M HCl

Time, h	Mean \pm SE	Difference from initial	Individual totals	Variance analysis source of variation	sum of squares	d.f.	mean squares	F
AChE								
Initial	9.8 \pm 0.919		76.4 36.3	times	10.53	2	5.265	15.26
1.5	11.0 \pm 1.040	1.5**	38.1 23.0	indiv	71.99	5	14.398	41.73
3	11.1 \pm 0.766	1.6**	28.2 37.9	error	3.45	10	0.345	
				total	85.97	17		
pH								
Initial	7.44 \pm 0.036		22.05 22.41	times	0.029	2	0.0145	8.53
1.5	7.37 \pm 0.031	0.07*	22.27 21.91	indiv	0.050	5	0.0100	5.88*
3	7.35 \pm 0.033	0.09**	22.21 22.09	error	0.017	10	0.0017	
				total	0.096	17		
$p\text{CO}_2$								
Initial	31.9 \pm 0.944		91.9 90.8	times	142.87		71.435	11.1
1.5	28.6 \pm 1.735	3.3*	80.0 95.4	indiv	143.05	5	28.610	4.44
3	25.0 \pm 1.735	6.9*	84.6 70.3	error	64.36	10	6.436	
				total	390.28	17		

Footnotes and explanations as in table I.

intervals were always carried out. Comparisons between initial and subsequent values were performed by Duncan's test. Results and statistical evaluations are shown in tables.

Erythrocyte AChE activity related to the hemoglobin concentration, significantly increases in the groups of animals subjected to various kinds of hypoxia (table I) such an increase is already present at 0.75 h in the bled or CoCl_2 -treated animals and at 1.5 h in the rabbits kept in a hypobaric chamber. The enzyme activity returns to normal values within 4.5 h in the CoCl_2 -treated animals, whereas in the other groups it still shows high values at 12 h.

Also the animals subjected to infusion with acids (table II III) show – within the considered time limits – increases of the erythrocyte AChE activity that are detectable at 1.5 and 3 h; moreover they show significant decreases of the $p\text{CO}_2$, whereas an acidotic state with significant decrease

Table IV Reticulocyte levels¹ observed before the beginning and at the end of the experiment in rabbits bled (I) kept in hypobaric chamber (II) CoCl_2 treated (III) and subjected to infusion of 0.1 N lactic acid (IV) or HCl (V)

Time, h	Mean \pm SE	Individual totals	Variance analysis source of variation	sum of squares	d.f	mean squares	F
<i>I</i>							
Initial	2.13 ± 0.167	4.3 4.6	times	0.167	1	0.167	<1
12	2.15 ± 0.143	3.2 3.7	indiv	137.500	5	27.500	18.75**
		5.3 4.6	error	7.333	5	1.467	
			total	145.000	11		
<i>II</i>							
Initial	2.07 ± 0.099	4.4 4.7	times	1.333	1	1.333	1
12	2.13 ± 0.131	3.4 3.7	indiv	74.000	5	14.800	11.1
		4.5 4.7	error	6.667	5	1.333	
			total	81.000	11		
<i>III</i>							
Initial	2.23 ± 0.158	4.9 4.4	times	6.833	1	6.833	<1
1	2.08 ± 0.164	3.6 3.8	indiv	80.500	5	16.100	1.09
		4 5.0	error	73.667	5	14.733	
			total	161.000	11		
<i>IV</i>							
Initial	1.95 ± 0.133	4.9 3.7	times	1.667	1	1.667	1.9
3	2.02 ± 0.108	3.7 4.1	indiv	84.00	5	16.800	19.34
		4.4 3.2	error	4.333	5	0.867	
			total	90.000	11		
<i>V</i>							
Initial	2.08 ± 0.140	5.1 3.8	times	1.333	1	1.333	1.43
3	2.02 ± 0.125	3.2 3.9	indiv	101.000	5	20.200	21.65
		4.3 4.3	error	4.667	5	0.933	
			total	107.000	11		

For further details and abbreviations see table I

The reticulocyte level is expressed as a reticulocyte/erythrocyte percentage.

of the blood pH appears only in the HCl-treated rabbits (group V table III)

The various groups of rabbits show no significant difference between reticulocyte counts carried out before the beginning and at the end of the various experiments (table IV)

Discussion

The results obtained in the course of the present studies supply new data about the erythrocyte AChE activity in particular experimental conditions: they are nevertheless hard to explain above all because the biological role of the enzyme studied remains still obscure.

First of all, the lack of significant variations in the reticulocyte levels leads us to exclude that the enzyme activity increases observed during the various experiments may be due to an increased reticulocyte release into the blood circulation.

Our recent studies [1] showed that high blood lactic acid levels with transient metabolic acidosis appear within the first 12 h in bled or CoCl_2 -treated rabbits. A strong increase of the blood lactic acid concentration is also observed as a consequence of hypobaric hypoxia: such an increase is, however, associated with respiratory alkalosis. The constant presence of increased blood lactic acid levels in the various experimental conditions achieved led us to verify whether the intravenous infusion of lactic or other acid gave rise to similar increases in the erythrocyte AChE activity. The positive results of such experiments and a total evaluation of the results obtained both in the present research and in the course of the previous studies concerning the regulation of the acid base balance [1] lead us to believe it probable that the observed increases of enzyme activity are not connected with particular changes of the blood pH. In fact, those increases occur both in the presence of acidosis, compensated or not, and of alkalosis. A significant decrease of the blood pCO_2 is the sole factor always present in the various conditions achieved. This decrease takes place both in the animals subjected to various hypoxic stresses, and in those subjected to acid infusion. Such results lead us to suggest the existence of a connection between the above-mentioned increases of erythrocyte AChE activity and the pCO_2 decrease.

References

1. AMBROSIO, M. V., PRINCIPITO, G. B., GRAMSI, G., DYLLAGATA, M., and GIOVANNINI, E. Acid-base balance changes and erythropoietin production in the early stages of hypoxia or after CoCl_2 treatment in the rabbit. *Br. J. Haemat.* (submitted for publication, 1977).
2. AUDITORI, J. V. and HARTMAN, R. C. Paroxysmal nocturnal hemoglobinuria. II. Erythrocyte acetylcholinesterase defect. *Am. J. Med.* 27: 401-410 (1959).

Table IV Reticulocyte levels¹ observed before the beginning and at the end of the experiment in bled (I), kept in hypobaric chamber (II) CoCl₂ treated (III) and subjected to infusion of 0.1 N lactic acid or HCl (V)

Time, h	Mean \pm SE	Individual totals	Variance analysis		d.f	mean squares	F
			source of variation	sum of squares			
<i>I</i>							
Initial	2.13 \pm 0.167	4.3 4.6	times	0.167	1	0.167	< 1
12	2.15 \pm 0.143	3.2 3.7	indiv	137.500	5	27.500	18.7
		5.3 4.6	error	7.333	5	1.467	
			total	145.000	11		
<i>II</i>							
Initial	2.07 \pm 0.099	4.4 4.7	times	1.333	1	1.333	1
12	2.13 \pm 0.131	3.4 3.7	indiv	74.000	5	14.800	11.1
		4.5 4.7	error	6.667	5	1.333	
			total	82.000	11		
<i>III</i>							
Initial	2.23 \pm 0.158	4.9 4.4	times	6.833	1	6.833	< 1
12	2.08 \pm 0.162	3.6 3.8	indiv	80.500	5	16.100	10
		4.2 5.0	error	73.667	5	14.733	
			total	161.000	11		
<i>IV</i>							
Initial	1.95 \pm 0.133	4.9 3.7	times	1.667	1	1.667	1.9
3	2.02 \pm 0.108	3.7 4.1	indiv	84.00	5	16.800	19.36
		4.2 3.2	error	4.333	5	0.867	
			total	90.000	11		
<i>V</i>							
Initial	2.08 \pm 0.140	5.1 3.8	times	1.333	1	1.333	1.43
3	2.02 \pm 0.125	3.2 3.9	indiv	101.000	5	20.200	1.65
		4.3 4.3	error	4.667	5	0.933	
			total	107.000	11		

For further details and abbreviations see table I

¹ The reticulocyte level is expressed as a reticulocyte/erythrocyte percentage.

of the blood pH appears only in the HCl treated rabbits (group V table III)

The various groups of rabbits show no significant difference between reticulocyte counts carried out before the beginning and at the end of the various experiments (table IV)

Preliminary Evidence for the Common Origin of a Lympho-Myeloid Complex in Man

RONALD D. BARR and JESSIE WATT

Haematology and Cytogenetic Units, University of Aberdeen Foresterhill, Aberdeen

Key Words: Human Lympho-haemopoietic Stem Cells

Abstract. In 2 patients with chronic myeloid leukemia, the Philadelphia chromosome was demonstrated in peripheral blood lymphocytes. This finding points to the common origin of lymphocytes and other blood cells in man.

It is assumed that, in man, lymphocytes are derived from a different progenitor cell than that from which all the other blood cells arise [11]. The object of the present study was to refute this supposition by examining the tissue distribution of the naturally occurring marker for human haemopoietic cells – the Philadelphia (Ph⁺) chromosome – in selected patients with chronic myeloid leukemia (CML).

Materials and Methods

Heparinized samples of peripheral venous blood were obtained from 2 patients with CML in partial remission (table I). Mononuclear cell concentrates were prepared by density sedimentation on Ficoll-Hypaque gradients [6] and depleted subsequently of monocytes by filtration through columns of cotton wool [3]. The resulting suspensions of lymphocytes were cultured with and without phytohemagglutinin (PHA) for 72 h and harvested by the method of MOORHEAD *et al.* [17]. In addition to standard aceto-orcein staining, chromosomes were subjected to G banding as described by SARKISOWITZ [21], with the substitution of detergent ('Lipcol') for trypsin [23]. The Ph⁺ chromosome was identified positively only in cells on which clear

- 3 BHATNAGAR S. P. The relationship between red cell cholinesterase levels and erythropoiesis induced by haemorrhage and cobalt treatment in rabbits. *Archs Int Pharmacodyn. Théor* 174 422-439 (1968)
- 4 ELLMAN G. L., COURTNEY K. D., ANDRIS, V. Jr and FEATHERSTONE R. M. A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochem Pharmacol* 7 88-95 (1961)
- 5 HERZ F., KAPLAN E., and SCHLYT E. S. Red cell acetylcholinesterase deficiency in autoimmune hemolytic anemia and in paroxysmal nocturnal hemoglobinuria. *Clinica chim Acta* 35 301-306 (1972)
- 6 HERZ F., KAPLAN E., and SCHLYT E. S. Red cell acetylcholinesterase deficiency in ABO hemolytic disease of the newborn. *Clinica chim Acta* 36 537-54 (1972)
- 7 HERZ, F. and KAPLAN E. *In vitro* modifications of red cell acetylcholinesterase activity. *Br J Haemat.* 26 165-178 (1974).
- 8 KRANTZ S. B. and JACOBSON L. O. Erythropoietin and the regulation of erythropoiesis (University of Chicago Press, Chicago 1970).
- 9 TIMORLING, E. B. and EARLEY A. J. The effect of some erythropoietic agents on the tissue tension of oxygen. *Br J Haemat.* 23 483-490 (1972)

Preliminary Evidence for the Common Origin of a Lympho-Myeloid Complex in Man

RONALD D. BARR and JESSIE WATT

Haematology and Cytogenetic Units, University of Aberdeen Foresterhill, Aberdeen

Key Words. Human Lympho-hemopoietic Stem Cells

Abstract In 2 patients with chronic myeloid leukemia, the Philadelphia chromosome was demonstrated in peripheral blood lymphocytes. This finding points to the common origin of lymphocytes and other blood cells in man.

It is assumed that, in man, lymphocytes are derived from a different progenitor cell than that from which all the other blood cells arise [11]. The object of the present study was to refute this supposition by examining the tissue distribution of the naturally occurring marker for human hemopoietic cells – the Philadelphia (Ph⁺) chromosome – in selected patients with chronic myeloid leukemia (CML).

Materials and Methods

Heparinized samples of peripheral venous blood were obtained from 2 patients with CML in partial remission (table I). Mononuclear cell concentrates were prepared by density sedimentation on Ficoll-Hypaque gradients [6] and depleted subsequently of monocytes by filtration through columns of cotton wool [3]. The resulting suspensions of lymphocytes were cultured with and without phytohemagglutinin (PHA) for 72 h and harvested by the method of MOORHEAD *et al.* [17]. In addition to standard aceto-orcein staining, chromosomes were subjected to G banding as described by SZABO *et al.* [21], with the substitution of detergent ('Lapsof') for trypsin [23]. The Ph⁺ chromosome was identified positively only in cells on which clear

Table I

Patient	Sex	Year of birth	Date of diagnosis	Date of study	Therapy	Total WBC count $\times 10^9/l$	Immature granulocytes,
R. C.	M	1955	July 1975	July 1976	busulphan	50.0	4
J. C.	F	1923	Feb. 1975	Nov. 1976	busulphan dlbromo- mannitol	7.9	7

decision could be made. Cells were not evaluated further if the chromosomes were damaged or poorly spread.

Results

Similar data were obtained from both patients. In the lymphocyte concentrates myelocytes accounted for less than 5% of the total cell number. No attempt was made to further separate the lymphocyte subpopulations. The lymphocyte cultures without PHA contained very few mitoses, all of which were Ph positive. These were probably the product of the small numbers of contaminating myelocytes. By comparison all cultures grown in the presence of PHA revealed that well over one third of the mitoses harbored the marker chromosome. Moreover the PHA-stimulated cultures showed an enormous increment in the mitotic index, reflecting the fact that the cell suspensions were lymphocyte-enriched (table II) for in these stimulated cultures more than 99% of the mitotic figures were devoid of cytoplasmic granules. An example of a Ph positive lymphocyte from patient R. C. is illustrated in figure 1.

Discussion

As one proceeds up the evolutionary scale there is progressive anatomical dissociation of lymphatic and blood-forming tissues, although functional interrelationships between the hemopoietic and immune systems persist [22, 30]. Thus in the phylogenetic advance from fish to man the role of the spleen in physiological hemopoiesis diminishes and this function becomes localized in and restricted to bone marrow.



Fig 1 The Philadelphia chromosome in PHA-stimulated lymphocytes from patient R. C. Aceto-orcein staining. *b* G-banding.

In mice it can be shown, by the use of the spleen colony assay and the T6 chromosome marker that the hemopoietic stem cell (HSC) gives rise not only to erythrocytes, granulocytes and megakaryocytes but also to lymphocytes [29]. In man there is persuasive if inconclusive evidence for the existence of circulating HSC, including the original observations of

Table I

Patient	Sex	Year of birth	Date of diagnosis	Date of study	Therapy	Total WBC count $\times 10^9/l$	Immature granulocytes, %
R. C.	M	1955	July 1975	July 1976	busulphan	50.0	4
J. C.	F	1923	Feb. 1975	Nov. 1976	busulphan dlbromo- mannitol	7.9	7

decision could be made. Cells were not evaluated further if the chromosomes were damaged or poorly spread.

Results

Similar data were obtained from both patients. In the lymphocyte concentrates myelocytes accounted for less than 5% of the total cell number. No attempt was made to further separate the lymphocyte subpopulations. The lymphocyte cultures without PHA contained very few mitoses, all of which were Ph positive. These were probably the product of the small numbers of contaminating myelocytes. By comparison all cultures grown in the presence of PHA revealed that well over one third of the mitoses harbored the marker chromosome. Moreover the PHA-stimulated cultures showed an enormous increment in the mitotic index reflecting the fact that the cell suspensions were lymphocyte-enriched (table II) for in these stimulated cultures more than 99% of the mitotic figures were devoid of cytoplasmic granules. An example of a Ph positive lymphocyte from patient R. C. is illustrated in figure 1.

Discussion

As one proceeds up the evolutionary scale there is progressive anatomical dissociation of lymphatic and blood-forming tissues, although functional interrelationships between the hemopoietic and immune systems persist [22, 30]. Thus in the phylogenetic advance from fish to man the role of the spleen in physiological hemopoiesis diminishes and this function becomes localized in and restricted to bone marrow.

will be mainly premorbid, having entered the circulation before the onset of the disease, and accordingly will not exhibit the Ph chromosome.

Two important modifications were therefore employed in the present study: lymphocytes were selectively concentrated from patients in established remission, so enhancing the opportunity of identifying the marker chromosome in these cells. The results are consistent with the predicted coexistence of morbid ('Ph positive') and premorbid ('Ph negative') lymphocyte populations. It is of further interest in this regard that the patient J C., who had a longer duration of established disease had a greater fraction of 'Ph positive' lymphocytes.

There is no selective removal of major lymphocytic subpopulations during density sedimentation in Ficoll-Hypaque [13-28] and filtration through cotton wool [3] and in a mixed population of T and B cells blastogenesis is induced in both by PHA [12]. Although it is possible, nevertheless, that, in the present study the Ph chromosome occurred in only one population of lymphocytes, this seems highly unlikely since the precursors of both T and B cells are demonstrable in human bone marrow [1, 4, 18, 25] and these may therefore be expected to share a common origin.

Thus the present findings, albeit derived from only two patients, do suggest that, in man, as in lower animals, lymphocytes arise from the same progenitor cells as give rise to the other formed elements of the blood.

Acknowledgements

We wish to thank Dr AUDREY A. DAWSON for allowing us to study these patients and Dr BARBARA M. PAGE for helpful discussion. This study was supported by SRC research studentship B/74/208 to J W.

References

- 1 ARDOU, N. L., ALAVI, J. B. and ARDOU, N. L. Human bone marrow lymphocytes. B and T cell precursors and subpopulations. *Blood* 47: 423-430 (1976).
- 2 BARR, R. D. and FALKOW, P. J. Clonal origin of chronic myelocytic leukemia. *New Engl. J. Med.* 289: 307-309 (1973).
- 3 BARR, R. D. and PERRY, E. Lymphocyte purification from normal human peripheral blood by filtration through columns of cotton wool. *Scand. J. Haematol.* 17: 300-304 (1976).

Table II Culture of CML lymphocytes

Patient	Fold increment in MI with PHA	Ph negative fraction, %	Ph positive fraction
R. C	214	64	36
J. C	93	56	44

temporary engraftment when patients with marrow hypoplasia received transfusions of peripheral blood leucocyte concentrates [7 15 20]. Moreover our recent *in vivo* culture studies have identified a likely candidate for the role of HSC in man [5] – the large null lymphocyte isolated from normal human peripheral blood.

Based on the principle of random X-chromosome inactivation in female somatic cells [16] our earlier studies of CML were in black African women who were heterozygous for the common variants of the X linked enzyme glucosyl-6-phosphate dehydrogenase. It was demonstrated thereby that CML is a disease of clonal origin – it arises from the malignant transformation of a single cell [2]. That cell is probably an HSC, for it is established that the Ph chromosome is present in cells of the erythrocytic, megakaryocytic and monocytic series as well as in developing granulocytes [9 10 19 24 26 27].

In these latter reports it was deduced that the Ph chromosome was not present in the lymphocytes in CML on the basis of the failure to produce an increment in Ph positive mitoses when unseparated CML cells were cultured with PHA. An exceptional case was recorded by KEMP *et al* [14] the Ph chromosome was first detected in and confined to the patient's marrow cells, while 9 months later 89% of PHA stimulated cells from unseparated peripheral blood revealed the marker chromosome. At the time of the latter sample the differential lymphocyte count was 40%. The obvious implication of these findings appears to have evaded the authors.

Such studies were marred by two fundamental flaws which relate to the fact that the investigations were undertaken usually early in the disease often at initial diagnosis when the disease was of course in relapse. The differential lymphocyte count is low in this circumstance so a detectable increment in the mitotic index of unseparated CML cells cultured with PHA is unlikely to occur. Furthermore since the majority of circulating lymphocytes are long lived [8] those present at diagnosis in CML

- brates; in *GORDON Regulation of hematopoiesis*, vol. 1, pp. 67-76 (Appleton-Century-Crofts, New York 1970).
- 22 STURTEWANT, G. Mammalian chromosomes G banded in 4 minutes. *Genetics* (in press, 1977).
- 23 TOUNG, I. M., JACOB, F. A., COURT-BROWN, W. M., BAKER, A. G., and WILLIAMSON, E. R. D. Cytogenetic studies on bone marrow in chronic myeloid leukemia. *Lancet* *i* 844-846 (1963).
- 24 TOURADOU, J. L., DUCOFF, G. S., TOURADOU, F. RHO, Y. M., and GOOD, R. A. Differentiation of human bone marrow cells into T lymphocytes by *in vitro* incubation with thymic extracts. *Clin. exp. Immunol.* *17* 151-158 (1971).
- 25 TRUTTELLO, J. M. and OMOTO, S. Chromosomal alteration of erythropoietic cells in chronic myeloid leukemia. *Acta haemat.* *29* 311-316 (1963).
- 26 WRAJDA, J., FRIED, E., TRO, J. H., CARBONE, P. P. and BAILEY, O. The distribution of the Philadelphia chromosome in patients with chronic myelogenous leukemia. *Blood* *22* 664-673 (1963).
- 27 WHITFIELD, T. L., BERARDI, R. S., and RABIN, B. S. Quantitation of human peripheral blood T and B lymphocytes. *Int. Archs Allergy appl. Immun.* *48* 731-738 (1975).
- 28 WU, A. M., TELL, J. E., SOMMERFELT, L., and MCCULLOCH, E. A. Cytological evidence for relationship between normal hematopoietic colony-forming cells and cells of the lymphoid system. *J. exp. Med.* *127* 455-463 (1968).
- 29 YOFFEY, J. M. *Quantitative cellular hematology* (Thomas, Springfield 1967).

- 4 BARR, R. D., SARIN, P., and PERRY S. Terminal transferase in human bone marrow lymphocytes. *Lancet* *i*. 508-509 (1976)
- 5 BARR, R. D., WILANO-PENG J. and PERRY S.. Hemopoietic stem cells in human peripheral blood. *Science* *190* 284-285 (1975).
- 6 BOYUM, A.. Separation of leucocytes from blood and bone marrow. *Scand. J. clin. Lab. Invest.* *21* suppl. 97 (1968).
- 7 BRONSON W. R., MCGHEE, M. H. and MORSE, E. E.. Hemopoietic graft detected by a change in ABO group. *Blood* *23* 239-249 (1964)
- 8 BUCKTON, K. E., COURT-BROWN, W. M. and SMITH, P. G. Lymphocyte survival in men treated with x-rays for ankylosing spondylitis. *Nature, Lond.* *214* 470-473 (1967)
- 9 CHERVENICK, P. A., ELLIS, L. D., PAN, S. F., and LAWSON A. L.. Human leukemic cells *in vitro* growth of colonies containing the Philadelphia (Ph) chromosome. *Science* *174* 1134-1136 (1971)
- 10 CLEIN, O. P. and FLEISMAN, R. J.. Involvement of the erythroid series in blastic crisis of chronic myeloid leukemia. Further evidence for the presence of Philadelphia chromosome in erythroblasts. *Br J Haemat.* *12* 754-758 (1966).
- 11 ELVER, M. W. and ISRAEL, M. C. G. Cytogenetic studies in unusual forms of chronic myeloid leukemia. *Acta haemat.* *35* 129-141 (1967)
- 12 GREAVES, M. F., JAMOSY G. and DOENIGT M. Activation of human T and B lymphocytes by polyclonal mitogens. *Nature, Lond.* *243* 698-701 (1974)
- 13 HOLM, G., PETTERSON, D., MELLSTEDT H., HEDGECOCK, E., and BLOTH, B.. Lymphocyte subpopulations in peripheral blood of healthy persons. Characterization by surface markers and lack of selection during purification. *Clin. exp. Immunol.* *30* 443-457 (1975)
- 14 KEMP N. H., STAFFORD J. L., and TAYLOR, R.. Chromosome studies during early and terminal chronic myeloid leukemia. *Br. med. J.* *i* 1010-1014 (1964).
- 15 LEVIN, R. H., WILANO J., TITO, J. H., CARBONE, P. F., FREI, E., and FREUDENBERG, E. J. Persistent mitoses of transfused homologous leukocytes in children receiving anti-leukemic therapy. *Science* *142* 1305-1311 (1963)
- 16 LYON M. F. Gene action in the X chromosome of the mouse. *Nature, Lond.* *190* 372-373 (1961)
- 17 MOORHEAD P. S., NOWELL, P. C., MILLMANN, W. J., BATTIPS, D. M., and HUNTERFORD D. A. Chromosome preparations of leukocytes cultured from human peripheral blood. *Expl Cell Res.* *20* 613-616 (1960).
- 18 PYKE, K. W. and GELFAND, E. W. Morphological and functional maturation of human thymic epithelium in culture. *Nature, Lond.* *251* 421-423 (1974).
- 19 RASTRICK, J. M., FITZGERALD, P. H., and GUNZ, F. W. Direct evidence for presence of Ph¹ chromosome in erythroid cells. *Br. med. J.* *i* 96-98 (1974)
- 20 SCHWARZENBERG L., MATHIE, G., AMIEL, J. L., CATTAN A., SCHNEIDER, M., and SCHLUMBERGER, J. R.. Study of factors determining the usefulness and complications of leukocyte transfusions. *Am. J. Med.* *43* 206-213 (1967).
- 21 SEABRIGHT M.. A rapid banding technique for human chromosomes. *Lancet* *ii* 971-972 (1971)
- 22 SEDGAL, C. B. Possible hematopoietic mechanisms in non-mammalian verte

Over the past year we have taken the opportunity to study rosette formation in 13 patients with CLL at intervals during the course of their illness either before treatment, or during intermittent therapy. In addition, we have studied these cells after incubation and washing procedures.

Materials and Methods

Subjects Studied

Normal values for T, B and M rosetting cells were obtained from the peripheral blood of over 50 healthy donors.

The leukaemic patients were in two groups, 10 untreated subjects who were clinically well, and 3 patients requesting treatment or who had received previous therapy and were again on treatment. Of the latter 2 subjects had been studied first in the untreated group and treatment was for troublesome lymphadenopathy in one instance and persistent splenic discomfort in the other.

Sample Preparation

Blood was collected into heparinized BSS and layered onto a Ficoll-Trisoll mixture [6]. After centrifugation, cells from the interface were collected and washed once in BSS. The cells were adjusted to a count of $4 \times 10^6/\text{ml}$.

Sheep erythrocytes. A volume of washed packed red cells was incubated with 3 vol of papain (1% papain and 0.4% L-cysteine hydrochloride dissolved in buffered saline of pH 5.4) : dilution of 1 in 10 in PBS at 37 °C for 15 min. The cells were then washed 3 times in PBS and for E rosettes 0.1 ml packed red cells was resuspended in 10 ml of BSS.

Mouse red blood cells (MRBC). Fresh blood was collected into citrated saline and washed 3 times. 0.1 ml of packed red cells was resuspended in 10 ml of BSS.

E and M rosettes. T (0.25 ml) of lymphocyte suspension was added 0.05 ml fetal calf serum and 0.25 ml of appropriate red cell suspension in a tube 12 x 75 mm. The tubes were centrifuged at 1,000 rpm for 2 min, and incubated on ice at 4 °C for 1 h. The rosettes were resuspended by gently rolling the tubes, and were counted using phase contrast microscopy. Any lymphocyte with 3 or more adhering red cells was considered rosette. A total of 100-200 lymphocytes was counted.

Complement-dependent (EAC) rosettes. A 5% suspension of washed sheep erythrocytes was made and 5 ml was mixed with an equal volume of 1/5,000 dilution of rabbit haemolytic serum in BSS. After incubation at 37 °C for 30 min, the cells were washed and resuspended in 10 ml. 0.25 ml fresh normal human serum was added and incubated further 30 min. After further washing the cells were resuspended in a volume of 10 ml BSS. For rosette formation, 0.5 ml of lymphocyte suspension $2 \times 10^6/\text{ml}$ was mixed with 0.5 ml of sensitized sheep cells, and incubated at 37 °C for 5 min. Following centrifugation at 1,000 rpm for 2 min, and incubation at room temperature for 30 min, the cells were vigorously resuspended using a rotomixer and rosettes counted as for E and M rosettes, using phase microscopy to exclude monocyte rosettes.

Significance of Mouse Red Cell Rosette-Forming Lymphocytes in Chronic Lymphocytic Leukaemia

G. D. PEGRUM and C. A. EVANS

Haematology Department, Charing Cross Hospital Medical School, London

Key Words Mouse red cell Rosettes · Chronic lymphatic leukaemia White blood count Washed cells

Abstract Increased mouse red cell (M) rosetting lymphocytes were demonstrated in the peripheral blood of patients with chronic lymphatic leukaemia. The range was wide, and patients showed considerable variation not only in the numbers of M cells but also in T and B rosetting lymphocytes. Treatment reduced M rosette lymphocytes proportionately as the total white count fell and differential removal occurred only when the patients became leucopaenic. If we assume the M rosetting cells are the abnormal 'leukaemic' cells, treatment does not preferentially remove these. The M rosetting capacity appeared to be related to the presence of an immunoglobulin factor previously demonstrated on the cells and in the serum of patients with CLL which enhances *in vivo* viability of the leukaemic cells.

Introduction

It has been established that a proportion of lymphocytes form rosettes with mouse red cells. In normal peripheral blood the numbers are small but in chronic lymphocytic leukaemia (CLL) rosettes are formed by many lymphocytes [15]. These M cells are immunoglobulin bearing and are thus considered to be a subpopulation of the increased numbers of B lymphocytes present in this condition [5-7]. The recognition of these cells has been regarded as a useful diagnostic procedure [3-14] and it has further been suggested that the numbers present correlate with the proliferative activity of the disease [1].

Table 17 Rosetting capacity of peripheral blood lymphocytes from patients with untreated chronic lymphatic leukaemia

Patient	Time interval from previous observation	Total lymphocyte count $10^9/l$	T total ($10^9/l$) %	B total ($10^9/l$) %	M total ($10^9/l$) %
1		120	8.4 (7)	10.8 (9)	51.6 (43)
	3/12	115	8.0 (7)	1.1 (1)	80.5 (70)
	3/12	125	2.3 (2)	1.2 (1)	38.7 (31)
2		48	2.4 (5)	3.8 (8)	7.2 (15)
	1/12	42	5.8 (14)	4.2 (10)	10.5 (25)
	5/12	46	7.3 (16)	2.7 (6)	17.9 (39)
	2/12	54	0.5 (1)	2.1 (4)	28.6 (53)
3		20	1.6 (8)	1.8 (9)	12.2 (61)
	2/12	22	1.1 (5)	4.4 (20)	11.8 (54)
	1/12	27	1.3 (5)	2.7 (10)	14.3 (53)
	1/12	25	1.2 (5)	2.2 (9)	8.5 (34)
	2/12	30	0.3 (1)	1.8 (6)	12.3 (41)
4		130	1.5 (1)	4.5 (3)	100.5 (67)
	3/12	219	1.0 (0.5)	4.3 (2)	118.2 (54)
	4/12	106	2.1 (2)	1.0 (1)	55.1 (52)
	1/12	200	4.0 (2)	2.0 (1)	156.0 (78)
5		15	0.9 (6)	0.4 (3)	3.3 (22)
	2/12	15	3.7 (25)	2.3 (17)	6.0 (40)
	3/12	19	1.9 (10)	2.8 (15)	3.2 (17)
	3/12	14	6.8 (49)	2.1 (15)	2.2 (16)
6		18	1.2 (7)	2.3 (13)	7.3 (41)
	3/12	20	0.1 (0.5)	0.6 (3)	7.6 (38)
	4/12	18	1.4 (8)	1.0 (6)	7.9 (44)
7		107	0.5 (0.5)	4.2 (4)	48.1 (45)
	3/12	18	1.0 (6)	1.4 (8)	2.8 (16)
	3/12	16	0.4 (3)	0.1 (1)	5.4 (34)
8		25	6.2 (25)	3.5 (14)	2.0 (8)
	2/52	24	2.4 (10)	1.6 (7)	7.4 (31)
	1/12	37	4.4 (12)	2.5 (7)	12.9 (35)
	1/12	55	2.2 (4)	0.5 (1)	13.7 (25)
	1/12	208	20.8 (40)	1.5 (3)	23.4 (45)
	3/12	107	1.0 (1)	1.0 (1)	17.1 (16)
	4/12	185	5.5 (3)	27.7 (15)	39.2 (32)

Washed CLL cells After harvesting lymphocytes from the Ficoll Trisoll mixture, the cells were washed a total of 6 times in Hanks BSS with one incubation of 30 min at 37 °C during the washing procedure.

Results

The percentages of T B and M rosette forming lymphocytes in the peripheral blood from 10 healthy individuals is shown in table I. In most cases, the total of rosetting lymphocytes is close to 100% indicating there are very few null or double rosetting cells.

Untreated Patients (table II)

Total white counts remain fairly steady in the majority of patients. Despite this, considerable variation was found in the proportion of lymphocytes assigned to each rosette group.

T rosettes Both the percentage and total numbers of T rosette-forming lymphocytes changed without any alteration in the patient's condition. Both higher and lower levels than the normal range were encountered without any clinical requirement for treatment.

EAC (B) rosettes Although the percentage of these is low the absolute number was raised above the normal level in the majority of patients, but fluctuations occurred. In patients 2, 3, 9 and 10 there appeared to be

Table I Normal donor rosetting lymphocytes (%)

	T	B	M	Total
1	67	36	3	106
2	54	38	1	93
3	60	30	1	91
4	67	26	3	96
5	61	36	1	101
6	62	33	0	95
7	66	43	4	113
8	53	17	5	75
9	51	29	4	84
10	88	11	1	120

T rosette-forming cells 55-75% $2.700-0.720 \times 10^9/l$ B rosette-forming cells 25-35% $1.230-0.160 \times 10^9/l$ M rosette-forming cells <1-5% $0.150-0.010 \times 10^9/l$.

Table 17 Rosetting capacity of peripheral blood lymphocytes from patients with untreated chronic lymphatic leukaemia

Patient	Time interval from previous observation	Total lymphocyte count $10^9/l$	T total ($10^9/l$) %	B total ($10^9/l$) %	M total ($10^9/l$) %
1		120	8.4 (7)	10.8 (9)	51.6 (43)
	3/12	115	8.0 (7)	1.1 (1)	80.5 (70)
	3/12	125	2.5 (2)	1.2 (1)	38.7 (31)
2		48	2.4 (5)	3.8 (8)	7.2 (15)
	1/12	42	5.8 (14)	4.2 (10)	10.5 (25)
	5/12	46	7.3 (16)	2.7 (6)	17.9 (39)
	2/12	54	0.5 (1)	2.1 (4)	28.6 (53)
3		20	1.6 (8)	1.8 (9)	12.2 (61)
	2/12	22	1.1 (5)	4.4 (20)	11.8 (54)
	1/12	27	1.3 (5)	2.7 (10)	14.3 (53)
	1/12	25	1.2 (5)	2.2 (9)	11.1 (44)
	2/12	30	0.3 (1)	1.8 (6)	12.3 (41)
4		150	1.5 (1)	4.5 (3)	100.5 (67)
	3/12	219	1.0 (0.5)	4.3 (2)	118.2 (54)
	4/12	106	2.1 (2)	1.0 (1)	55.1 (52)
	1/12	200	4.0 (2)	2.0 (1)	156.0 (78)
5		15	0.9 (6)	0.4 (3)	3.3 (22)
	2/12	15	9.7 (25)	2.5 (17)	6.0 (40)
	3/12	19	1.9 (10)	2.8 (15)	3.2 (17)
	3/12	14	6.8 (49)	2.1 (15)	2.2 (16)
6		18	1.2 (7)	2.3 (13)	7.3 (41)
	3/12	20	0.1 (0.5)	0.6 (3)	7.6 (38)
	4/12	18	1.4 (8)	1.0 (6)	7.9 (44)
7		107	0.5 (0.5)	4.2 (4)	48.1 (45)
	1/12	18	1.0 (6)	1.4 (8)	1.8 (16)
	3/12	18	0.4 (3)	0.1 (1)	5.4 (34)
8		25	6.2 (25)	3.5 (14)	2.0 (8)
	2/52	24	2.4 (10)	1.6 (7)	7.4 (31)
	1/12	37	4.4 (12)	2.5 (7)	12.9 (35)
	1/12	55	2.2 (4)	0.5 (1)	13.7 (25)
	1/12	52	20.8 (40)	1.5 (3)	23.4 (45)
	3/12	107	1.0 (1)	1.0 (1)	17.1 (16)
	4/12	185	5.5 (3)	7.7 (15)	39.2 (32)

Table II (continued)

Patient	Time interval from previous observation	Total lymphocyte count $10^9/l$	T total ($10^9/l$) %	B total ($10^9/l$)	M total ($10^9/l$) %
9		10	5.7 (57)	2.5 (25)	1.9 (19)
	3/12	8	2.0 (26)	1.9 (24)	2.2 (28)
	4/12	9	0.5 (6)	0.9 (11)	3.8 (43)
	2/52	10	1.5 (15)	1.1 (11)	5.2 (52)
10		20	3.8 (19)	3.4 (17)	15.6 (78)
	2/52	41	2.4 (6)	3.6 (9)	12.3 (30)
	2/52	14	3.2 (23)	2.2 (16)	4.2 (30)

the least variation whilst others, such as patient 8, showed sharp differences of 50-fold or more

M rosettes These were uniformly high. The numbers were maintained at approximately the same level in most patients but large swings occurred in patients 4 and 8.

The total percentage of rosetting cells also varied and once again we were unable to link this with any changing clinical parameters. Patients 1-6, 9 and 10 have no significant lymphadenopathy or splenomegaly, 2, 3 and 6 have significant lymphadenopathy and 3 and 8 considerable symptomless splenomegaly.

Treated Patients (table III)

The total white cell count was reduced by therapy.

T cell rosettes T cell rosettes varied during treatment but no consistent pattern emerged and results were not significantly different from those who were untreated.

B rosettes There was a tendency for these to fall in 4 of the 5 treated patients. Considerable variation, however, was observed during clinical improvement which was maintained in each patient.

M rosettes We only managed to reduce these to normal numbers in two of the five patients, both of whom became leucopenic. We are impressed by the fact that the fall in M cells in all the patients appears related to the total fall in lymphocytes (fig. 1). This suggests that treatment is

Table III. Rosetting capacity of peripheral blood lymphocytes from patients on treatment for CLL.

Patient	Time intervals from previous observation	Total lymphocyte count $10^9/l$	T total ($10^9/l$) %	B total ($10^9/l$) %	M total ($10^9/l$) %
1		50	7.0 (14)	4.5 (9)	0.5 (1)
	1/52	33	1.9 (6)	5.2 (16)	3.6 (11)
	1/52	18	2.7 (15)	1.9 (12)	6.1 (33)
	1/52	13	0.8 (7)	2.9 (23)	1.1 (9)
	9/12	14	0.5 (4)	5.8 (42)	0.9 (7)
2 (3)		30	0.3 (1)	1.8 (6)	12.3 (41)
	2/52	17	1.0 (6)	1.0 (6)	8.5 (50)
	3/52	27	1.3 (5)	2.4 (9)	13.2 (49)
	1/12	7	0.9 (13)	0.4 (7)	2.1 (41)
	2/12	10	2.0 (20)	1.4 (14)	2.8 (28)
	1/12	11	1.3 (12)	0.3 (3)	3.0 (28)
3		3	0.6 (22)	0.7 (26)	0.09 (3)
	2/12	4	1.3 (34)	1.0 (29)	0.9 (23)
	5/12	4	0.4 (12)	0.3 (8)	0.1 (3)
	1/12	2	0.3 (17)	0.7 (37)	0.01 (0.5)
	3/12	2	0.5 (25)	0.3 (17)	0.04 (2)
4 (2)		60	0.6 (1)	2.4 (4)	31.8 (53)
	1/52	113	3.3 (3)	11.3 (10)	45.2 (40)
	2/12	35	1.7 (5)	1.7 (5)	13.8 (38)
	1/12	17	1.1 (11)	1.7 (10)	1.1 (41)
5		61	10.3 (17)	14.6 (24)	22.5 (37)
	1/12	3	1.1 (38)	0.6 (20)	0.3 (11)
	2/52	4	1.3 (33)	0.4 (12)	0.02 (0.5)

Patient numbers in brackets refer to the same patient in the untreated group (table II).

not removing leukaemic cells preferentially if the M rosetting cells are considered to be the leukaemic population.

Cell Washing (table IV)

10 of 12 experiments showed a significant reduction in M rosetting capacity after the washing procedure in the other two only slight falls occurred. When we attempted to reconstitute the capacity to M rosette by incubation with serum we achieved very variable and inconsistent results.

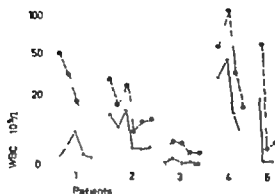


Fig 1 Comparison of total WBC count with the number of M rosette-forming lymphocytes in 5 treated patients. --- = Total WBC; — = total M rosetting cells.

Table 11 M rosetting capacity of leukaemic lymphocytes before and following washing procedure (%)

Unwashed control	Washed cells
16	0
30	—
33	6
28	8
43	0
34	1
30	17
17	6
49	29
10	4
57	31
2	0

Discussion

Our findings demonstrate considerable variation in the number of M rosette forming lymphocytes in the peripheral blood of patients with chronic lymphocytic leukaemia. In untreated subjects the rosette forming lymphocytes were significantly higher than in patients receiving therapy and both were higher than in normal individuals. This supports the suggestion of CATOVSKY *et al* [2] and MILLARD [11] that this parameter can be valuable in diagnosis. We were unable to substantiate the findings of BEAUMARLAGE and FOCAN [1] who studied the ratio of M to T rosette

forming cells. The use of ratios was not useful in our hands since although absolute numbers of M rosetting lymphocytes were lower in the treated individuals, the numbers of T and B rosetting cells also fell as the total leucocyte count was reduced. On treatment the M rosetting cells seemed to disappear from the peripheral blood in the same proportion as other lymphocytes relative to the falling white count. This suggests that we are not differentially removing the leukaemic cell population during therapy a view also expressed recently by MILLARD [11].

It is apparent that although we might consider M rosetting cells as abnormal leukaemic immunoglobulin-bearing lymphocytes, there are increased numbers of B rosetting cells which may be present either as part of the leukaemic process or because of it. T rosetting lymphocytes were variable and we were unable to corroborate the suggestions of CATOVSKY *et al.* [3] that high numbers of these are associated with an improved prognosis.

Incubation and washing of the leukaemic cells considerably reduces the M rosetting capacity. In previous studies we demonstrated that this procedure removes a factor which enhances the viability of the leukaemic lymphocytes [12] and that the collected washing medium is rich in immunoglobulin.

It has been suggested that the receptor for M rosettes is also an immunoglobulin [7] and this would be in accord with our finding if we assume this is removed by the washing procedures. We consider that significant numbers of leukaemic cells absorb immunoglobulin from the sera and that this imparts many of the abnormal features such as poor mitogenic responses [13] and altered cell kinetics [9]. The proportion of immunoglobulin present on the cells and in the serum may be more important than that present on the lymphocytes alone. We have found that the viability enhancing factor in serum is sometimes markedly reduced by treatment and can be altered by intercurrent disease. The significance, quantity and character of this immunoglobulin factor present in the serum and on the lymphocytes has yet to be determined together with its relevance to the disease process.

References

1. BRAUMARLACK, M. L. and FOCAN, C. Immunocytodherence of human lymphocytes in lymphoproliferative disorders. *IRCS med. Sci., Cancer, Cell membrane Biol. Haemat. Immun. Allergy* 3: 476 (1975).

- 2 CATOVSKY D., CHERCHI, M., OKOS, A., HEGDE, U. and GALTON, D. A. G. Mouse red-cell rosettes in B-lymphoproliferative disorders. *Br J Haemat.* 33 173-177 (1976)
- 3 CATOVSKY D., MILIANI, E., OKOS, A. and GALTON D. A. G.. Clinical significance of T-cells in chronic lymphocytic leukaemia. *Lancet* *ii*, 751 (1974)
- 4 DAVIS, S.. The variable pattern of circulating lymphocyte subpopulations in chronic lymphocytic leukaemia. *New Engl. J. Med.* 294 1150-1153 (1976)
- 5 DOBOZY A., HUEZ, S., HUNYADI, J. and SAROC, N. Formation of mouse erythrocyte rosettes by human lymphocytes. A B-cell marker. *Clin. exp. Immunol.* 23 382-384 (1976)
- 6 EVANS, C. A. and MIDDLETON V. L.. Evaluation of methods for preparing pure lymphocyte suspensions from peripheral blood. *J. clin. Path.* 23 822-823 (1970)
- 7 FORBES, I. J. and ZALEWICKI, P. D. A subpopulation of human B lymphocytes that rosette with mouse erythrocytes. *Clin. exp. Immunol.* 26 99-107 (1976)
- 8 GUPTA, S., GOOD R. A., and SIGUAL, F. P. Rosette-formation with mouse erythrocytes. II. A marker for human B and non T lymphocytes. *Clin. exp. Immunol.* 25 319-327 (1976).
- 9 LEWIS, C. M., EVANS, C. A., and FLORUM, G. D. Reduced rates of membrane turnover in lymphocytes from chronic lymphatic leukaemic patients. *Clin. exp. Immunol.* 23 219-227 (1976).
- 10 MUELLMAYER, G. and HUBER, H.. Receptor sites for complement on certain human peripheral blood lymphocytes. *J. Immun.* 105 670-676 (1970).
- 11 MILLARD R.. Mouse-red-cell rosetting test in chronic lymphocytic leukaemia. *Lancet* *ii*, 1204 (1976).
- 12 PERERA, D. J. B. and FLORUM, G. D. A factor causing enhanced viability of lymphatic leukaemic lymphocytes. *Acta haemat.* 52 273-279 (1974)
- 13 PERERA, D. J. B. and FLORUM, G. D. *In vitro* stimulation of chronic lymphocytic leukaemic lymphocytes. *Br J Haemat.* 27 289-296 (1974)
- 14 STATHOPOULOS, G. and DAVIES, A. J. S. Human lymphocytes and mouse red cells. *Lancet* *i*, 1078 (1976)
- 15 STATHOPOULOS, G. and ELLIOTT E. V.. Formation of mouse or sheep red blood cell rosettes by lymphocytes from normal and leukaemic individuals. *Lancet* *i* 600-601 (1974).

Megakaryocyte Polyploidization in May Hegglin Anomaly¹

M. MAYER, H. SPERLING, J. SCHAEFER and W. QUEISSER

Department of Internal Medicine, Faculty of Clinical Medicine Mannheim,
University of Heidelberg, Heidelberg

Key Words. Megakaryocyte polyploidization May-Hegglin anomaly Cytophotometry Autoradiography

Abstract. Polyploidization of megakaryocytes was studied in bone marrow aspirates from 3 patients with May Hegglin anomaly by combined application of cytophotometric determination of the DNA content and autoradiography with ³H-TdR labeling *in vitro*. A marked elevation of the influx of progenitor cells into the megakaryocytic cell system as well as decreased maturation capacity from type II to type III megakaryocytes was observed possibly contributing to the pathological platelet sequestration. The polyploidization activity as assessed by ³H TdR labeling and nuclear DNA content was normal.

Introduction

The megakaryocytes of the bone marrow do not show mitotic cell divisions as seen in all other hemopoietic cell lines, but undergo continuous polyploidization up to 32c in man. Another specific phenomenon is the microtubular demarcation system, which enables cytoplasm to release platelets. The mode of polyploidization has been the subject of many investigations during the last years. Evidence has been provided for alternation of DNA synthesis and periods of rest suggesting a rhythmical polyploidization [1]. It may be assumed that the pattern of polyploidization influences platelet maturation and release. Therefore, it was of interest to

Supported in part by the Deutsche Forschungsgemeinschaft (Qu 33/1).

Received: August 5, 1977 accepted: October 30, 1977

- 2 CATOVSKY D, CHERCHI, M., OKOS, A., HEDDE, U and GALTON, D A G. Mouse red-cell rosettes in B-lymphoproliferative disorders. *Br J Haemat.* 33 173-177 (1976)
- 3 CATOVSKY D., MILLANI, E., OKOS, A., and GALTON D A. G.. Clinical significance of T-cells in chronic lymphocytic leukaemia. *Lancet* *ii* 751 (1974).
- 4 DAVIE, S. The variable pattern of circulating lymphocyte subpopulations in chronic lymphocytic leukaemia. *New Engl. J Med.* 294 1150-1153 (1976).
- 5 DOMOZY A., HUKZ, S., HUNYADI, J and SIMON, N. Formation of mouse erythrocyte rosettes by human lymphocytes. A B-cell marker. *Clin. exp Immunol.* 23 382-384 (1976)
- 6 EVANS, C. A. and MIDDLETON, V L. Evaluation of methods for preparing pure lymphocyte suspensions from peripheral blood. *J clin. Path.* 23 822-823 (1970)
- 7 FORBER, I J and ZALEWSKI, P D.. A subpopulation of human B lymphocytes that rosette with mouse erythrocytes. *Clin. exp Immunol.* 26. 99-107 (1976)
- 8 GUPTA, S., GOOD, R. A., and SIEGAL, F P.. Rosette-formation with mouse erythrocytes. *II. A marker for human B and non T lymphocytes. Clin. exp Immunol.* 25 319-327 (1976)
- 9 LEWIS, C. M., EVANS, C. A., and PEORUM G D. Reduced rates of membrane turnover in lymphocytes from chronic lymphatic leukaemic patients. *Clin. exp Immunol* 23 219-227 (1976)
- 10 MICHELMAYER, G and HUBER, H. Receptor sites for complement on certain human peripheral blood lymphocytes. *J Immun.* 105 670-676 (1970).
- 11 MILLARD, R.. Mouse-red-cell rosetting test in chronic lymphocytic leukaemia. *Lancet* *ii* 1204 (1976).
- 12 PERERA, D J B and PEORUM G D. A factor causing enhanced viability of lymphatic leukaemic lymphocytes. *Acta haemat.* 52 273-279 (1974)
- 13 PERERA, D J B and PEORUM, G D. *In vitro* stimulation of chronic lymphocytic leukaemic lymphocytes. *Br J Haemat.* 27 289-296 (1974).
- 14 STATHOPOULOS, G and DAVIES, A. J S.. Human lymphocytes and mouse red cells. *Lancet* *i* 1078 (1976).
- 15 STATHOPOULOS, G and ELLIOTT E. V. Formation of mouse or sheep red blood cell rosettes by lymphocytes from normal and leukaemic individuals. *Lancet* *i* 600-601 (1974).

The extinction was measured by scanning automatically as large as possible square or rectangle within the nucleus. The relative DNA content ($AU = \text{arbitrary units}$) of individual nuclei was calculated from $AU = E \times A$ ($E = \text{extinction}$, $A = \text{nuclear area}$). The nuclear areas were determined by planimetry.

For determination of the diploid standard, the DNA content of about 50 lymphocytes was measured. From the lymphocytic diploid standard the different accumulations of DNA values of the megakaryocytes could be attributed to the polyploidy stages 4c, 8c, 16c, etc. Further the DNA content of unlabeled megakaryocytes at 8c or 16c was averaged, and from this value the megakaryocytic diploid standard (2c) was calculated.

Autoradiographs of the Feulgen-stained smears were made by the dipping film technique using Ilford K2 liquid emulsion. The smears were exposed for 14–22 days. For background correction the number of silver grains overlying the megakaryocyte nuclei was drawn in diagram against the nuclear size. The smallest megakaryocytes with more than 12 grains and megakaryocytes double that size with more than 24 grains were designated as labeled. More details of the combined cytophotometric and autoradiographic technique were described earlier [11].

Cytology / Megakaryocytes

According to the paucic staining properties, megakaryocytes were divided into three groups as described by BASSIS [2] and modified by FERNANDEZ *et al.* [4].

Type I Megakaryocytes with distinct cytoplasmic basophilia, no granulation and high nuclear-cytoplasmic ratio.

Type II Megakaryocytes with basophilic cytoplasm, but slightly amorphous granulation.

Type III Megakaryocytes showing no basophilia, but strong amorphous granulation of the cytoplasm and low nuclear-cytoplasmic ratio. Since the nuclear size is well correlated to the DNA content [11], but not to the state of maturation, this criterion of cell differentiation was discarded.

Results

Frequency of Megakaryocytes in Each Morphologic Compartment

The data of the megakaryocyte differentiation of all cases studied were compared to those obtained from normal individuals (table II). A marked relative increase of type II and a decrease of type III was observed. The proportion of type I was similar to that found in normal cases. The ratio of type I:II:III showed a shift to the left from 1:1.3:5 in normal individuals to 1.3:1.5:1.7, 1.2:2.4 and 1.4:6:1.3 respectively.

Megakaryocyte Ploidy Pattern

The distribution of the relative DNA values and ^3H TdR-labeled cells in each morphological cell compartment of case 1 is given in figure 1.

study the megakaryocyte polyploidization in some inborn errors of the platelet system

The May Hegglin anomaly is an autosomal-dominant bleeding disorder characterized by giant platelets inclusions within the granulocytic cell line (Döhle bodies) and commonly thrombocytopenia and minor hemorrhagic manifestations [6-10]. Up to 1974 83 cases were described [5]. The study was performed on 3 patients with May Hegglin anomaly reported by LECHNER *et al* [9].*

Materials and Methods

Patients

The personal data and platelet counts at the time of investigation are given in table 1. Hemorrhagic manifestations were seen only in case 3 who showed mild epistaxis. Cases 1 and 2 showed no bleeding tendency so far.

General Procedure

The marrow was aspirated into a syringe containing 0.5 ml 1% Na₂EDTA in 0.9% NaCl and incubated for 1 h with tritiated thymidine (³H TdR 2 μ Ci/ml specific activity 2 Ci/mmol) at room temperature. Smears were made from the marrow fragments and stained with Pappenheim stain. The individual cells were marked and photographed for subsequent localization, for consecutive cytophotometry and autoradiography and for the determination of nuclear size by planimetry. Upon completion of the photography Pappenheim stain was leached out by treatment with 50% ethanol for 5-10 min.

The slides were restained by the Feulgen method with hydrolysis for 12 min in N HCl at 60 °C. Pararosaniline was applied for Schiff's reagent. The staining time was 60 min.

Cytophotometry and Autoradiography

For cytophotometric determination of the DNA content of megakaryocytes, a Cytoscan (Zeiss, BRD) was used. Monochromatic light at 570 nm was employed.

Table 1 Personal data and platelet counts of the 3 patients

Diagnosis	Case No.	Initials	Sex	Age, years	Platelets/ μ
May	1	Su. E.	M	39	114,000
Hegglin	2	Su. A.	F	15	134,000
anomaly	3	Su. R.	M	1	110,000

* We thank Prof. Dr. K. BARDON, Frankfurt, for his help to study some members of the previously described family.

Table III Percentage distribution of the different megakaryocyte types in rest (G phases) and in DNA synthesis (S phase)

Case		G ₁ phase (4c)	G ₂ phase (8c)	G ₁ phase (16c)	G phase (32c)	G phase (64c)	S phase	U
<i>Type I</i>								
1	117	0.9	41.0	21.4	4.3	0	29.9	2.6
2	107	0	30.8	23.4	0.9	0	43.0	1.9
3	104	1.9	27.9	31.7	1.9	0	34.6	1.9
Normal								
	Mean	3.5	14.2	30.4	5.7	0	43.7	2.5
	Range	1.6	11.9	25.0	1.2	0	33.9	1.2
		-7.1	-17.3	-38.3	-8.6		-53.6	-4.8
<i>Type II</i>								
1	142	0	18.3	50.7	21.1	0	8.2	0.7
2	150	0	23.3	53.3	13.3	0	7.3	2.7
3	130	0.8	17.7	60.0	11.5	0	7.7	2.3
Normal								
	Mean	0	11.7	51.1	18.1	0	17.5	1.6
	Range	0	10.6	44.3	8.5	0	13.2	0
			-12.3	-57.0	-27.4		-26.8	-2.7
<i>Type III</i>								
1	109	0.9	12.8	50.5	34.9	0.9	0	0
2	112	0	32.1	62.5	5.4	0	0	0
3	100	0	31.0	56.0	11.0	0	1.0	1.0
Normal								
	Mean	0.5	9.8	58.4	25.0	0	1.8	4.5
	Range	0	4.0	48.1	19.3	0	0	2.4
		-1.2	-13.2	-63.3	-32.1		-3.2	-6.6

Cases 1-3 May-Hegglin anomaly. Normal values from Quintana et al. [11]. = Number of cells assessed. U = unlabeled cells between the different polyploidy classes.

Mean and SD of 5 normals.

few cells were seen in 32c. In types II and III the highest proportion was apparent in 16c. The ³H-TDR-labeled cells were distributed within as well as between the polyploidy stages, representing the cells in DNA synthesis. In type III active DNA synthesis was not observed. A similar polyploidization pattern was found in cases 2 and 3.

The percentages of megakaryocytes in the different polyploidy stages and in the S phase are summarized in table III. As compared with the

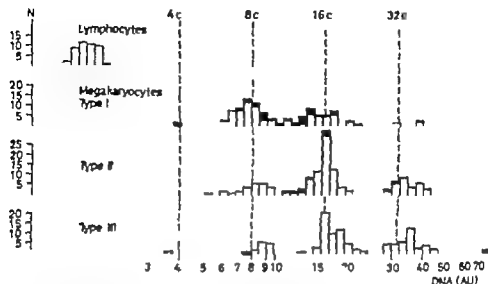


Fig 1 Relative DNA content in arbitrary units (AU) and ³H TdR labeling (shaded areas) of megakaryocytes in May Hegglin anomaly (case 1) N = Number of cells assessed 4c, 8c, 16c, 32c = polyploidy stages.

Table II Percentage distribution of megakaryocytes type I II and III within 500 megakaryocytes

Diagnosis	Case	Type I %	Type II %	Type III
May-Hegglin anomaly	1	17.2	56.8	26.0
	2	9.4	68.0	22.6
	3	14.6	66.8	18.6
Normal (n = 5) ¹	mean	18.4	17.3	67.7
	range	15.0-22.3	15.4-21.1	56.5-69.5

¹ QUESSER *et al* [11].

The diploid standard calculated from the megakaryocytes (2c) was somewhat different from that of lymphocytes as observed earlier [11]. However the ploidy values derived from unlabeled 16c megakaryocytes (2c, 4c, 8c, etc.) corresponded exactly to the accumulations of different degrees of DNA values. The polyploidy stages of type I megakaryocytes extended from 4c to 32c, and of type II and III mostly from 8c to 32c. In type I the most impressive accumulation was seen on the 8c level and only a

Table III Percentage distribution of the different megakaryocyte types in rest (G phases) and in DNA synthesis (S phase)

Case	n	G phase (4c)	G ₂ phase (8c)	G phase (16c)	G phase (32c)	S phase (64c)	S phase	U
<i>Type I</i>								
1	117	0.9	41.0	21.4	4.3	0	29.9	2.6
2	107	0	30.8	23.4	0.9	0	43.0	1.9
3	104	1.9	27.9	31.7	1.9	0	34.6	1.9
Normal								
	Mean	3.5	14.2	30.4	5.7	0	43.7	2.5
	Range	1.6	11.9	25.0	1.2	0	33.9	1.2
		-7.1	-17.3	38.3	-8.6		-53.6	-4.8
<i>Type II</i>								
1	142	0	18.3	50.7	21.1	0	9.2	0.7
2	150	0	23.3	53.3	13.3	0	7.3	2.7
3	130	0.8	17.7	60.0	11.5	0	7.7	2.3
Normal								
	Mean	0	11.7	51.1	18.1	0	17.5	1.6
	Range	0	10.6	44.3	6.5	0	13.2	0
			-12.3	-37.0	-27.4		-26.8	-2.7
<i>Type III</i>								
1	109	0.9	12.8	50.5	34.9	0.9	0	0
2	112	0	32.1	64.5	4.4	0	0	0
3	100	0	31.0	56.0	11.0	0	1.0	1.0
Normal								
	Mean	0.5	9.8	58.4	25.0	0	0	4.5
	Range	0	4.0	48.1	19.3	0	0	2.4
		-1.2	-13.2	-63.3	-37.1		-3.4	-6.6

Cases 1-3 May-Hegglin anomaly. Normal values from Quenqua *et al.* [11]. n = Number of cells assessed. U = unlabeled cells between the different polyploidy classes.

Mean and SD of 5 normals

few cells were seen at 32c. In types II and III the highest proportion was apparent at 16c. The ³H TDR-labeled cells were distributed within as well as between the polyploidy stages, representing the cells in DNA synthesis. In type III active DNA synthesis was not observed. A similar polyploidization pattern was found in cases 2 and 3.

The percentages of megakaryocytes in the different polyploidy stages and in the S phase are summarized in table III. As compared with the

normal the amount of unlabeled 8c megakaryocytes of all types was markedly increased in each case. However the percentages of megakaryocytes in the higher polyploidy classes did not differ from the normal values. The ^3H TdR labeling of type I was only diminished in case 1 (29.9%) whereas in type II the labeling index was markedly decreased in all 3 cases.

Discussion

The literature concerning the pathogenesis of the May Hegglin anomaly deals mainly with the mechanism of platelet production and sequestration. Giant platelets have already been observed within the megakaryocytes [6-8]. The total peripheral platelet mass is regarded to be normal [1, 5]. Therefore it was postulated that the underlying defect of this disease is a disturbance of megakaryocyte maturation.

The results of the present study of megakaryocytopoiesis in May Hegglin anomaly may be summarized as follows: (1) Differentiation counting reveals a marked difference of the proportion of megakaryocytes in the different cytological maturation classes compared to the normal (table II). (2) Within the type I megakaryocytes the number of cells with an 8c DNA content is considerably increased. (3) The relative size of all type II compartments is strikingly enlarged as shown in figure 2. (4) The polyploidization pattern of megakaryocytes is normal as seen in a considerable amount of 32c cells (table III). (5) The ^3H TdR labeling index suggests a normal polyploidization activity of type I and a reduced one of type II (table III).

How may these data be understood and does the observed polyploidization and maturation pattern contribute to the understanding of the pathogenesis of the disease? The enlarged 8c type I compartment seen in all three patients indicates an enhanced influx of progenitor cells into the megakaryocytopoiesis. A growth of the compartment of 4c resting cells would be also expected but these young cells are difficult to identify cytologically. The normal ^3H TdR labeling of the type I megakaryocytes reflects a normal polyploidization activity of these cells. In contrast to the results obtained in normal individuals, the amount of type I megakaryocytes does not show further enlargement of the proportion of resting cells beyond the 8c level (fig. 2). This means that the further polyploidization of type I is accompanied by considerable cytoplasmic maturation leading to an increased size of the type II compartment.

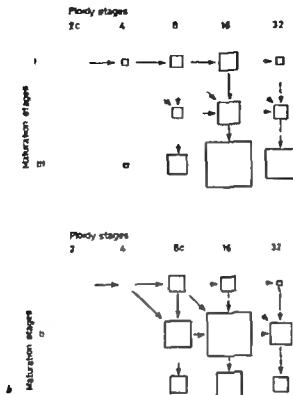


Fig. 2. Polyploidization and maturation scheme of megakaryocytes in normal individuals (a) and in May Hegglin anomaly (b). The size of the single squares represents the percentages of megakaryocytes types I, II and III in the different polyploidy stages calculated from the cell number (table II) and the proportion of cell at the different ploidy levels (table III). Mean values of 5 normal cases (II) and 3 cases with May Hegglin anomaly.

However the striking increase of the remaining cells of type II (Fig. 2) cannot be fully explained by this influx. The total of type II and III megakaryocytes in May Hegglin anomaly is similar to the normal conditions it seems to be likely that the further cytoplasmic maturation from type II to type III is disturbed. Additionally the polyploidization capacity of type II megakaryocytes is decreased as seen by a lowered ^3H TdR labeling index (table III).

normal the amount of unlabeled 8c megakaryocytes of all types was markedly increased in each case. However the percentages of megakaryocytes in the higher polyploidy classes did not differ from the normal values. The ^3H TdR labeling of type I was only diminished in case 1 (29.9%) whereas in type II the labeling index was markedly decreased in all 3 cases.

Discussion

The literature concerning the pathogenesis of the May Hegglin anomaly deals mainly with the mechanism of platelet production and sequestration. Giant platelets have already been observed within the megakaryocytes [6-8]. The total peripheral platelet mass is regarded to be normal [1-5]. Therefore, it was postulated that the underlying defect of this disease is a disturbance of megakaryocyte maturation.

The results of the present study of megakaryocytopoiesis in May Hegglin anomaly may be summarized as follows: (1) Differentiation counting reveals a marked difference of the proportion of megakaryocytes in the different cytological maturation classes compared to the normal (table II). (2) Within the type I megakaryocytes the number of cells with an 8c DNA content is considerably increased. (3) The relative size of all type II compartments is strikingly enlarged as shown in figure 2. (4) The polyploidization pattern of megakaryocytes is normal as seen in a considerable amount of 32c cells (table III). (5) The ^3H TdR labeling index suggests a normal polyploidization activity of type I and a reduced one of type II (table III).

How may these data be understood, and does the observed polyploidization and maturation pattern contribute to the understanding of the pathogenesis of the disease? The enlarged 8c type I compartment seen in all three patients indicates an enhanced influx of progenitor cells into the megakaryocytopoiesis. A growth of the compartment of 4c resting cells would be also expected but these young cells are difficult to identify cytologically. The normal ^3H TdR labeling of the type I megakaryocytes reflects a normal polyploidization activity of these cells. In contrast to the results obtained in normal individuals, the amount of type I megakaryocytes does not show further enlargement of the proportion of resting cells beyond the 8c level (fig. 2). This means that the further polyploidization of type I is accompanied by considerable cytoplasmic maturation leading to an increased size of the type II compartment.

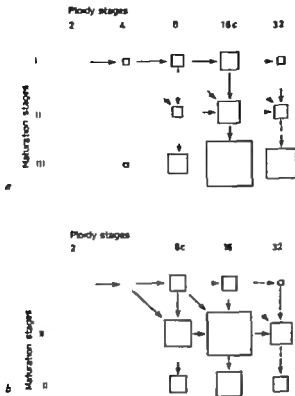


Fig. 2 Polyploidization and maturation scheme of megakaryocytes in normal individuals (a) and in May-Hegglin anomaly (b). The size of the single squares represents the percentages of megakaryocytes types I, II and III in the different polyploidy stages calculated from the cell number (table II) and the proportion of cells at the different ploidy levels (table III). Mean values of 5 normal cases [11] and 3 cases with May-Hegglin anomaly.

However the striking increase of the remaining cells of type II (Fig. 2) cannot be fully explained by this influx. The total of type II and III megakaryocytes in May-Hegglin anomaly is similar to the normal conditions, it seems to be likely that the further cytoplasmic maturation of type II to type III is disturbed. Additionally the polyploidization capacity of type II megakaryocytes is decreased as seen by a lowered ^3H TdR labeling index (table III).

References

- 1 BEHRENS, W. E. von: Evidence of phylogenetic canalization of the circulating platelet mass in man. *Thromb. Diath. haemorrh.* 27: 159-172 (1972).
- 2 BESSIS, M.: Cytology of the blood and blood forming organs (Grune & Stratton, New York 1956).
- 3 DÖHLE, V.: Leukozyteneinschlüsse bei Scharlach. *Zentrbl. Bakt. Parasitkde* 61: 63-68 (1911).
- 4 FLINENDIEN, L. E., ODARTCHENKO, N., COTTER, H. and BOND, V. P.: Kinetics of megakaryocyte proliferation. *Proc. Soc. exp. Biol. Med.* 3: 177-182 (1962).
- 5 GOODWIN, H. A. and GINSBURG, A. D.: May Hegglin anomaly: A defect in megakaryocyte fragmentation? *Br. J. Haemat.* 26: 117-128 (1974).
- 6 HEGGELIN, R.: Gleichzeitige konstitutionelle Veränderungen an Neutrophilen und Thrombozyten. *Helv. med. Acta* 12: 439-440 (1945).
- 7 JORDAN, S. W. and LARSEN, W. E.: Ultrastructural studies of the May Hegglin anomaly. *Blood* 25: 921-932 (1935).
- 8 KASE, L.: Megakaryocytes in May Hegglin anomaly. *Archs. Path.* 98: 11-113 (1974).
- 9 LECHNER, K., BREIDEN, K., MOSER, L., STOCKMOSER, L. und WENZEL, L.: May Hegglin'sche Anomalie: Beschreibung einer neuen Familie und Untersuchungen zur Funktion, Biochemie und Ultrastruktur der Thrombozyten. *Acta haemat.* 4: 303-320 (1969).
- 10 MAY, R.: Leukozyteneinschlüsse. *Dtsch. Arch. klin. Med.* 96: 1-6 (1909).
- 11 QUEISSER, U., QUEISSER, W. and SPIERTZ, B.: Polyploidization of megakaryocytes in normal humans, in patients with idiopathic thrombocytopenia and with pernicious anemia. *Br. J. Haemat.* 20: 489-501 (1971).

Platelet Aggregation in Diabetic Retinopathy

DRAGA CRETER, FIDA PAVLOTZKY and HANNA SAVIR

Departments of Haemostasis and Ophthalmology Hasharon Hospital, Petah Tikva

Key Words. Platelet aggregation Diabetic retinopathy Adenosine diphosphate Epinephrine

Abstract Platelet aggregation in diabetic retinopathy was investigated in a group of 25 patients. An enhanced activity induced by epinephrine and arachidonic acid was found in this group as compared with the controls, whereas in adenosine diphosphate (ADP) platelet aggregation no differences were observed. Spontaneous aggregation was found in 8% of the diabetic retinopathy cases.

Platelet aggregation with the classic substances adenosine diphosphate (ADP), epinephrine, thrombin, collagen and ristocetin has been described and is routinely used in hemostasis departments all over the world [3 11 17 18]. Recently the arachidonic acid and its action on platelet behavior and aggregation were studied [5 13 16]. Diabetic patients have been shown to have increased sensitivity of their platelets to ADP epinephrine and arachidonic acid [1 4-9 12, 15].

The aim of this study was to determine the platelet aggregation of patients with diabetic retinopathy when using ADP epinephrine and arachidonic acid.

Materials and Methods

The group of patients chosen for the laboratory hemostasis trial comprised 25 subjects with retinal findings compatible with diabetic retinopathy. 10 healthy blood bank donors were used as normal controls.

References

- 1 BEHRENS, W. E. von Evidence of phylogenetic canalisation of the circulating platelet mass in man *Thromb. Diath. haemorrh.* 27 159-172 (1972).
- 2 BESSIS, M. Cytology of the blood and blood forming organs (Grune & Stratton, New York 1956)
- 3 DÖHLE, V. Leukozyteneinschlüsse bei Scharlach. *Zentrbl. Bakt. Parasitkde* 67 63-68 (1911)
- 4 FLEVENDEGEN, L. E., ODARTSCHENKO, N., COTTIER, H. and BOND, V. P. Kinetics of megakaryocyte proliferation. *Proc. Soc. exp. Biol. Med.* 3 177-182 (1962)
- 5 GODWIN, H. A. and GINSBURG, A. D. May-Hegglin anomaly A defect in megakaryocyte fragmentation? *Br. J. Haemat.* 26 117-128 (1974)
- 6 HEGGLIN, R. Gleichzeitige konstitutionelle Veränderungen an Neutrophilen und Thrombozyten *Helv. med. Acta* 72 439-440 (1945)
- 7 JORDAN, S. W. and LARSEN, W. E. Ultrastructural studies of the May Hegglin anomaly *Blood* 25 921-932 (1935).
- 8 KASS, L. Megakaryocytes in May Hegglin anomaly *Archs. Path.* 98 112-113 (1974).
- 9 LECHNER, K., BREIDEN, K., MOSER, L., STOCKINGER, L. und WENZEL, L. May-Hegglin'sche Anomalie Beschreibung einer neuen Familie und Untersuchungen zur Funktion, Biochemie und Ultrastruktur der Thrombozyten. *Acta haemat.* 42 303-320 (1969).
- 10 MAY, R. Leukozyteneinschlüsse. *Dt. Arch. klin. Med.* 96 1-6 (1909).
- 11 QUEISSER, U., QUEISSER, W. and SPIERTZ, B. Polyploidization of megakaryocytes in normal humans, in patients with idiopathic thrombocytopenia and with pernicious anaemia *Br. J. Haemat.* 20 489-501 (1971)

Platelet Aggregation in Diabetic Retinopathy

DRAGA CRETER, FIRA PAVLOTZKY and HANNA SAVITZ

Departments of Haemostasis and Ophthalmology Hasharon Hospital, Petah Tikva

Key Words: Platelet aggregation Diabetic retinopathy Adenosine diphosphate Epinephrine

Abstract. Platelet aggregation in diabetic retinopathy was investigated in a group of 21 patients. An enhanced activity induced by epinephrine and arachidonic acid was found in this group as compared with the controls, whereas in adenosine diphosphate (ADP) platelet aggregation no differences were observed. Spontaneous aggregation was found in 8% of the diabetic retinopathy cases.

Platelet aggregation with the classic substances adenosine diphosphate (ADP) epinephrine, thrombin, collagen and ristocetin has been described and is routinely used in hemostasis departments all over the world [3 11 17 18]. Recently the arachidonic acid and its action on platelet behavior and aggregation were studied [5 13 16]. Diabetic patients have been shown to have increased sensitivity of their platelets to ADP epinephrine and arachidonic acid [1 4-9 12, 15].

The aim of this study was to determine the platelet aggregation of patients with diabetic retinopathy when using ADP epinephrine and arachidonic acid.

Materials and Methods

The group of patients chosen for the laboratory hemostasis trial comprised 25 subjects with retinal findings compatible with diabetic retinopathy. 10 healthy blood bank donors were used as normal controls.

The platelet aggregation was determined by the method of BORN and CROSS [2], using an Evans Electroselenium (EEL) aggregation meter model 169 with recorder. The platelet concentration was adjusted to $200,000 \pm 20,000/l$. Platelet-rich plasma (PRP) 0.6 ml and the following aggregating substances in a final concentration of 6.2×10^{-4} M ADP, 6×10^{-3} M epinephrine, 3μ M arachidonic acid were used. Platelet poor plasma was considered 100%, and PRP -0%. The maximal percentage of aggregation at 4 min was calculated. Before each determination, spontaneous aggregation was done in PRP of all patients and controls. The time for each platelet aggregation determination was 6 min.

Results and Discussion

Statistically determined platelet aggregation results presented no significant increased sensitivity to ADP that is 89.6 ± 4.92 in the normal group as compared with 86.5 ± 6.27 in the diabetic retinopathy patients. Enhanced activity by epinephrine was 98.0 ± 3.65 in normals, as compared with 113.61 ± 3.31 in the patients.

Arachidonic acid platelet aggregation was obtained in 40% of the cases, as compared with the control group which presented no activity related to this substance. In 8% of the investigated cases with diabetic retinopathy spontaneous aggregation was also observed.

Platelets from diabetic subjects synthesize PGE_2 in response to ADP, epinephrine, collagen and arachidonic acid [5]. It is suggested [5, 14] that in diabetes an increased prostaglandin synthetase activity exists and from this an enhanced platelet aggregation. Our results concerning the epinephrine and arachidonic acid platelet aggregation confirm these findings. In contrast, no different activity with ADP as compared with the normal group was found in this study.

Spontaneous aggregation not related to the onset of the disease or its severity can explain the tendency of some diabetic patients to develop thrombosis. An increased synthesis of thromboxane A₂ [10] or a decreased synthesis of prostacyclin [10] can be the cause.

For prediabetic states it is suggested to find a marker which will be useful to prevent the platelet abnormalities in subjects genetically predisposed to become diabetics.

References

1. BENSOUSSAN, D., LEVY TOLEDANO, S., PARRA, P., CAHN, J. and CANTVET, J. Platelet hyperaggregation in increased plasma level of von Willebrand factor in diabetics with retinopathy. *Diabetologia* 11: 307-312 (1975).

- 2 BORM, E V R. and CROSS, M. J. The aggregation of blood platelets. *J Physiol* 168: 178-195 (1963).
- 3 BORM, E V R. Current ideas on the mechanism of platelet aggregation. *Ann N Y Acad. Sci.* 201: 4-12 (1972).
- 4 COLWELL, J A., CHAMBERS, A., and LARVINS, M. Inhibition of labile aggregation-stimulating substance (LASS) and platelet aggregation in diabetes mellitus. *Diabetes* 24: 684-687 (1975).
- 5 COLWELL, J A., HALONKA, P V, SARI, K., LEVINE, J, SAGEL, J and NAM, P R. S. Altered platelet function in diabetes mellitus. *Diabetes* 25 suppl. 2, pp. 826-831 (1976).
- 6 COLWELL, J A., SAGEL, J, CROOK, L, CHAMBERS, A., and LARVINS, M. Correlation of platelet aggregation plasma factor activity and megathrombocytes diabetic subjects with and without vascular disease. *Metabolism* 26: 279-285 (1977).
- 7 HEATH, H., BRIDGES, W D., CANEVER, J V, POLLOCK, J, HUNTER, P R., KELLEY J and BLOOM, A. Platelet adhesiveness and aggregation in relation to diabetic retinopathy. *Diabetologia* 7: 308-313 (1971).
- 8 KWAAN, H. C., COLWELL, J A., CRUZ, S., SUWANWELA, N and DOBBIE, J G. Increased platelet aggregation in diabetes mellitus. *J Lab. clin. Med.* 80: 236-246 (1972).
- 9 KWAAN, H. C., COLWELL, J A., and SUWANWELA, N. Disseminated intravascular coagulation in diabetes mellitus, with reference to the role of increased platelet aggregation. *Diabetes* 21: 108-113 (1972).
- 10 Editorial. Prevention of thrombosis. *Lancet* 127: 128 (1977).
- 11 ROSS, E. C. and GREEN, D. Disorders of platelet function. *Med Clin No Am.* 56: 35-46 (1972).
- 12 SAGEL, J, COLWELL, J A, CROOK, L., and LARVINS, M. Increased platelet aggregation in early diabetes mellitus. *Ann intern. Med.* 82: 733-738 (1975).
- 13 SILVER, M J, SMITH, J B, INGERMAN, C., and KOCHE, J J. Arachidonic acid induced human platelet aggregation and prostaglandin formation. *Prostaglandins* 4: 863-875 (1973).
- 14 SMITH, J B, INGERMAN, C, KOCHE, J J and SILVER, M. J. Formation of prostaglandins during the aggregation of human blood platelets. *J clin. Invest.* 52: 965-969 (1973).
- 15 SZIKSZY, M. Platelet aggregation in diabetes mellitus. *Adv Cardiol* vol. 4, pp 179-186 (Karger Basel 1970).
- 16 VARGAS, B B. and ZIMMER, P. Platelet aggregation induced by arachidonic acid is accompanied by release of potential inflammatory mediators distinct from PGE₂ and PGF. *Nature new Biol* 244: 114-116 (1973).
- 17 VERWYLEN, J, GAETANO, G. DE, and VERSTRAETE, M. Round-the-table conference on normal and modified platelet aggregation. *Acta med scand.* suppl. 525 (1971).
- 18 WEISS, H J. Thrombocytopathia. *Clin Haemat.* 1: 369-381 (1971).

The platelet aggregation was determined by the method of BORN and CROSS [2], using an Evans Electroselenium (EEL) aggregation meter model 169 with recorder. The platelet concentration was adjusted to $700,000 \pm 70,000/\mu\text{L}$. Platelet-rich plasma (PRP) 0.6 ml and the following aggregating substances in a final concentration of 6.2×10^{-4} M ADP, 6×10^{-3} M epinephrine, 3 M arachidonic acid were used. Platelet poor plasma was considered 100%, and PRP - 0%. The maximal percentage of aggregation at 4 min was calculated. Before each determination, spontaneous aggregation was done in PRP of all patients and controls. The time for each platelet aggregation determination was 6 min.

Results and Discussion

Statistically determined platelet aggregation results presented no significant increased sensitivity to ADP that is 89.6 ± 4.92 in the normal group as compared with 86.5 ± 6.27 in the diabetic retinopathy patients. Enhanced activity by epinephrine was 98.0 ± 3.65 in normals, as compared with 113.61 ± 3.31 in the patients.

Arachidonic acid platelet aggregation was obtained in 40% of the cases, compared with the control group which presented no activity related to this substance. In 8% of the investigated cases with diabetic retinopathy spontaneous aggregation was also observed.

Platelets from diabetic subjects synthesize PGE_2 in response to ADP, epinephrine, collagen and arachidonic acid [5]. It is suggested [5, 14] that in diabetes an increased prostaglandin synthetase activity exists and from this an enhanced platelet aggregation. Our results concerning the epinephrine and arachidonic acid platelet aggregation confirm these findings. In contrast, no different activity with ADP as compared with the normal group was found in this study.

Spontaneous aggregation not related to the onset of the disease or its severity can explain the tendency of some diabetic patients to develop thrombosis. An increased synthesis of thromboxane A_2 [10] or a decreased synthesis of prostacyclin [10] can be the cause.

For prediabetic states it is suggested to find a marker which will be useful to prevent the platelet abnormalities in subjects genetically predisposed to become diabetics.

References

1. BENBOUSSIAN, D., LEVY TOLEDANO, S., PASSA, P., CAEN, J. and CANTVET, J. Platelet hyperaggregation in increased plasma level of von Willebrand factor in diabetics with retinopathy. *Diabetologia* 11: 307-312 (1975).

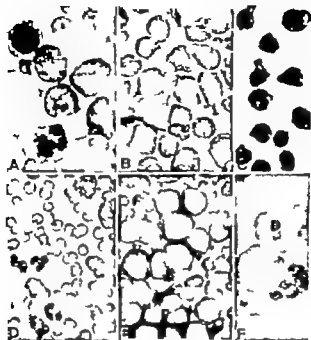


Fig 1 Blood (A) and bone marrow (B) at presentation, CSF sediment (C) blood (D) and bone marrow (E) in chronic myeloid phase bone marrow (F) in terminal crisis.

on the right side. Biopsy showed myeloid leukaemic infiltration. A cytogenetic study of the marrow revealed the Ph chromosome, the neutrophil alkaline phosphatase score resulted low.

After a few days the treatment was changed from busulfan + vincristine and prednisone owing to the changes in the blood picture: WBC $4 \times 10^9/l$ with 26% blasts, RBC $2.8 \times 10^{12}/l$, platelets $14 \times 10^9/l$. The marrow showed 90% undifferentiated blasts (fig 1F). Despite the new treatment the blast count increased and the patient died on 20-IV-1974 of cerebral haemorrhage.

In summary this is a case of CML, Ph positive, initiating with a clinical and haematological picture typical of an ALL with PAS-positive lymphoblasts and tumour-like infiltration of the breasts, very sensitive to ALL oriented chemotherapy. Concerning the combined occurrence of breast infiltration and CNS involvement, we have found it mentioned only in case No. 7 of PETERSON *et al* [4] whilst in case No. 3 and 7 of

Acta haemat 60 56-58 (1978)

Chronic Myeloid Leukaemia Initiating as Acute Lymphoid Leukaemia

To the Editor

In the last years some cases of chronic myeloid leukaemia (CML) initiating as acute lymphoid leukaemia (ALL) have been described [1-5]. A similar case was observed by us

C. P. a 37 year-old woman developed phlebitis shortly after her third delivery (12 IV 1973). This resolved quickly. She then developed bilateral mastitis, followed by pallor and weakness. Blood examination (9-V) showed: RBC $2.5 \times 10^{12}/L$, WBC $43 \times 10^9/L$, platelets $80 \times 10^9/L$. On admission the patient showed generalized lymphadenopathy, marked splenomegaly, bilateral enlargement of the breasts, which contained hard, painful nodules, skin dimpling and blushing. The leucocyte count rose to $72 \times 10^9/L$, with 40% lymphoblast-like cells (fig. 1A), but there were also 7% myelocytes, 3% metamyelocytes and 2% basophils. The bone marrow was hypercellular with total replacement by PAS-positive lymphoblasts (fig. 1B). After two courses of COAP she entered complete remission: RBC $3.8 \times 10^{12}/L$, WBC $4.5 \times 10^9/L$ with 72% neutrophils, platelets $240 \times 10^9/L$, bone marrow hypocellular with 3% blasts. The breast infiltration, lymphadenopathy and splenomegaly were resolved.

In November despite further treatment with COAP and POMP she showed a CML like picture: WBC $2.6 \times 10^9/L$ with 6% blasts, 18% granulocyte precursors, marrow hypercellular with 10% myeloblasts and 49% granulocyte precursors (fig. 1C, D). She had a good response to 6-MP: WBC $11 \times 10^9/L$ with 1% blasts, 6% granulocyte precursors, platelets $950 \times 10^9/L$, hypercellular bone marrow with myeloblasts 1% and granulocyte precursors 30%.

In February she was readmitted because of meningeal involvement and splenomegaly. She was in haematological relapse with WBC $95 \times 10^9/L$ with 6% blasts and 74% granulocyte precursors. The lumbar puncture showed increased pressure and CSF sediment composed of many lymphoblast like cells (fig. 1E). The meningeal involvement resolved after intrathecal methotrexate and cranial irradiation. February 11 she was started on busulfan, but a few days later the breast infiltration recurred.

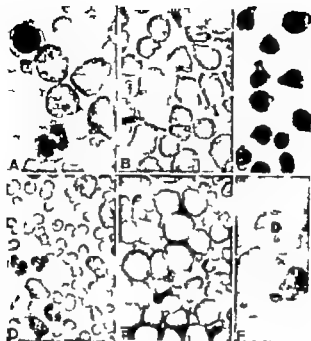


Fig 1 Blood (A) and bone marrow (B) at presentation CSF sediment (C); blood (D) and bone marrow (E) in chronic myeloid phase bone marrow (F) in terminal crisis.

on the right side biopsy showed myeloid leukaemic infiltration. A cytogenetic study of the marrow revealed the Ph chromosome the neutrophil alkaline phosphatase score resulted low.

After few days the treatment was changed from busulfan to vincristine and prednisone owing to the changes the blood picture WBC $4 \times 10^9/l$ with 36% blasts; RBC $2.8 \times 10^{12}/l$, platelets $14 \times 10^9/l$. The marrow showed 90% undifferentiated blasts (fig 1F). Despite the new treatment the blast count increased and the patient died on 20-IV 1974 of cerebral haemorrhage.

In summary this is a case of CML, Ph positive, initiating with a clinical and haematological picture typical of an ALL with PAS-positive lymphoblasts and tumour like infiltration of the breasts, very sensitive to ALL oriented chemotherapy. Concerning the combined occurrence of breast infiltration and CNS involvement, we have found it mentioned only in case No. 7 of PETERSON *et al* [4] whilst in case No 3 and 7 of

Acta haemat. 60 36-38 (1978)

Chronic Myeloid Leukaemia Initiating as Acute Lymphoid Leukaemia

To the Editor

In the last years some cases of chronic myeloid leukaemia (CML) initiating as acute lymphoid leukaemia (ALL) have been described [1-5]. A similar case was observed by us.

C. P., a 37 year-old woman developed phlebitis shortly after her third delivery (12 IV 1973). This resolved quickly. She then developed bilateral mastitis, followed by pallor and weakness. Blood examination (9-V) showed RBC $2.5 \times 10^{12}/l$, WBC $43 \times 10^9/l$, platelets $80 \times 10^9/l$. On admission the patient showed generalized lymphadenopathy, marked splenomegaly, bilateral enlargement of the breasts, which contained hard, painful nodules, skin dimpling and blushing. The leucocyte count rose to $72 \times 10^9/l$, with 40% lymphoblast-like cells (fig. 1A) but there were also 2% myelocytes, 3% metamyelocytes and 2% basophils. The bone marrow was hypercellular with total replacement by PAS-positive lymphoblasts (fig. 1B). After two courses of COAP she entered complete remission. RBC $3.8 \times 10^{12}/l$, WBC $4.5 \times 10^9/l$ with 72% neutrophils, platelets $240 \times 10^9/l$, bone marrow hypocellular with 3% blasts. The breast infiltration, lymphadenopathy and splenomegaly were resolved.

In November despite further treatment with COAP and POMP she showed a CML-like picture. WBC $26 \times 10^9/l$ with 6% blasts, 18% granulocyte precursors, marrow hypercellular with 10% myeloblasts and 49% granulocyte precursors (fig. 1C, D). She had a good response to 6-MP. WBC $11 \times 10^9/l$ with 1% blasts, 6% granulocyte precursors, platelets $930 \times 10^9/l$, hypercellular bone marrow with myeloblasts 1% and granulocyte precursors 30%.

In February she was readmitted because of meningeal involvement and splenomegaly. She was in haematological relapse with WBC $95 \times 10^9/l$ with 6% blast and 74% granulocyte precursors. The lumbar puncture showed increased pressure and CSF sediment composed of many lymphoblast-like cells (fig. 1E). The meningeal involvement resolved after intrathecal methotrexate and cranial irradiation. February 11 she was started on busulfan, but a few days later the breast infiltration recurred.

Intrathecal Methotrexate Causing Paraplegia in a Middle-Aged Woman

To the Editor

The treatment and prophylactic therapy of meningeal leukemia with intrathecal (IT) methotrexate (MTX) in acute lymphoblastic leukemia is a well-established procedure. However attention must be paid to the increasing number of reports of serious complications due to IT MTX (1). The present work describes a 46-year-old woman suffering from acute lymphoblastic leukemia, who slowly developed progressive permanent flaccid paraplegia post MTX treatment and a rise in CSF protein level during that therapy.

Case Report

A 46-year-old woman with acute lymphoblastic leukemia received treatment with prednisone, 6 MP cytosine-araboside and later vincristine. Complete hematological and clinical remission was achieved.

Half a year later the patient complained of severe headache but neurological examination revealed no pathology. A lumbar puncture showed the presence of lymphoblasts in CSF. Five IT injections of 20 mg MTX with parabens preservative / 20 ml of normal saline were administered over a 2-week period. The lymphoblasts in CSF disappeared and the protein level rose from 80, 65, 135, to 400 mg/dl while the white blood cells changed from 58, 1, 113 to 60 / l. The patient received cranial irradiation of 2,400 rad.

1 day after the last IT MTX injection the patient began complaining of numbness and pains in both legs. Over a 3-week period she developed flaccid paraplegia with sensory level at D₁₂, accompanied by stool and urinary incontinence, and finally right arm weakness. Partial resolution of the neurological disturbances was observed in the next 6 months.

BEARD *et al* [1] and No 5 of PETERSON *et al* [4] CNS involvement alone was present.

The principal interest of such cases has been already outlined [2]

References

- 1 BEARD, M. E. J. DURRANT J., CATOVSKY B. WILTSHAW E. AMES, J. L. BREARLEY R. L. JURE, B. WRIGLEY P. F. M. JANOSSEY G. GREAVES, M. F. and GALTON, D. A. O. Blast crisis of chronic myeloid leukaemia (CML). I Presentation simulating acute lymphoid leukaemia (ALL) Br J Haemat. 34 167-178 (1976).
- 2 JANOSSEY G., ROBERTS, M. and GREAVES, M. F. Target cell in chronic myeloid leukaemia and its relationship to acute lymphoid leukaemia. Lancet ii. 1058-1061 (1976)
- 3 JENKINS, D. W. RIVERA H. P. and COLMAN, C. A. Case report. Acute lymphatic leukemia followed by a clinical picture indistinguishable from chronic granulocytic leukemia in the same patient. Am. J. med. Sci. 264 329-335 (1972).
- 4 PETERSON L. C. BLOOMFIELD C. D. and BRUNTING, R. D. Blast crisis as an initial or terminal manifestation of chronic myeloid leukemia. A study of 28 patients. Am J Med. 60 209-220 (1976).
- 5 TANZER, J., JACQUELLAT C. BOIRON M. et BERNARD J. Leucémie aigue lymphoblastique suivie trois ans plus tard d'une leucémie myéloïde chronique à chromosome Philadelphia Lyon méd. 233 311-315 (1975)

WALTER PAOLINO ALESSANDRO LEVIS, LUCIANO CARAMELLINO and FRANCO PAOLINO
Ospedale Maggiore S. G. Battista e Città di Torino, Ente regionale Torino (Italy)

tween proteins and cells may perhaps serve to warn the physician of impending trouble.

It is suggested that a worsening of the neurological condition, or an increase in CSF protein (protein cells dissociation) or pressure during IT MTX therapy be taken as an indication to delay further injection, in order to diminish or avoid severe neurological complications.

References

- 1 GAGLIANO, R. G. and COSTANZI, J. J. Paraplegia following intrathecal methotrexate. *Cancer* 37: 1663-1668 (1976).
- 2 DUTTERA, N. J. BLEYER, W. A. POMEROY, T. C. LEVENTHAL, C. M. and LEVENTHAL, B., Irradiation, methotrexate toxicity and the treatment of meningeal leukemia. *Lancet* ii: 703-707 (1973).
- 3 BLEYER, W. A. DRAKE, J. C. and CHAMBER, B. A. Neurotoxicity and elevated cerebrospinal-fluid methotrexate concentration in meningeal leukemia. *New Engl. J. Med.* 289: 770-773 (1973).
- 4 SHAPIRO, W. R., YOUNG, D. F. and MINTA, B. P. Methotrexate. Distribution in cerebrospinal-fluid after intravenous, ventricular and lumbar injections. *New Engl. J. Med.* 293: 161-166 (1975).
- 5 LUDDY, R. E. and GRIM, V. P. A. Paraplegia following intrathecal methotrexate. *J. Pediatr.* 83: 988-992 (1973).

S. WEISS and Y. KAPLAN, Roshach Hospital, Tel Aviv (Israel)

The patient died from septicemia after the reappearance of the leukemia. Permission for autopsy was denied.

Discussion

Paraplegia complicating IT MTX treatment has been reported mainly in children and teenagers and in a few adults [1]. The toxic neurological reaction to MTX therapy can be classified into three groups according to the clinical manifestations [2]. Chemical arachnoiditis, encephalopathy and myelopathy. In myelopathy direct damage to the spinal cord or root nerves may cause serious consequences such as paralysis, transient or permanent paraplegia, quadriplegia and even death. This complication may perhaps be partially preventable.

Different predisposing factors were suggested to be responsible for the development of these neurological complications [1]. These include the chemical preservatives, diluents and contaminants of MTX. Comparison of 5 patients with severe neurotoxic manifestations post IT MTX with 20 asymptomatic patients receiving the same therapy showed that the toxic patients were significantly older and had overt meningeal leukemia [3]. The same characteristics were found in our patient. Furthermore neurotoxic symptoms occurred in the presence of elevated CSF concentrations of MTX. Therefore, it is suggested that frequent monitoring of CSF MTX concentration may be predictive of serious neurotoxicity and drug dosage and drug interval be adjusted accordingly [3]. However neither the minimal effective concentration of MTX in CSF nor the toxic concentration is known [4] and therefore can not yet serve as a safe guide in treatment or prevention of the neurological complications.

In a recent review of the literature GAGLIANO and COSTANZI [1] found 10 cases of paraplegia following IT MTX therapy and added 1 CSF examinations during IT MTX therapy have been reported in detail in only 2 patients [5].

The elevation of proteins in LUNDY's case [5] before the onset of paralysis was 33-75-500 mg/dl and in our case 80-60-135 mg/dl. Generally abnormal spinal fluid findings (proteins, cells) respond to IT MTX therapy by decreasing or disappearing. There was no other explanation for the rise of proteins in our case except MTX neurotoxicity as there was no evidence of infection (including TBC), hemorrhage, subarachnoid block (normal myelography) or even arachnoiditis. While there is no one specific reliable indicator of a possible neurotoxic reaction the discrepancy be-

tween proteins and cells may perhaps serve to warn the physician of impending trouble.

It is suggested that a worsening of the neurological condition, or an increase in CSF protein (protein cells dissociation) or pressure during IT MTX therapy be taken as an indication to delay further injection, in order to diminish or avoid severe neurological complications.

References

- 1 GAOLIANO, R. E and CONYANZI, J. J. Paraplegia following intrathecal methotrexate. *Cancer* 37 1663-1668 (1976).
- 2 DUTTERA, N. J. BLEYER, W. A. POMEROY T. C. LEVENTHAL, C. M. and LEVENTHAL, B. Irradiation, methotrexate toxicity and the treatment of meningeal leukemia. *Lancet* ii 703-707 (1973).
- 3 BLEYER, W. A., DRAKE, J. C. and CHARNER, B. A. Neurotoxicity and elevated cerebrospinal-fluid methotrexate concentration in meningeal leukemia. *New Engl. J. Med.* 289 770-773 (1973).
- 4 SHAPIRO, W. R., YOUNG, D. F. and MEHTA, B. P. Methotrexate Distribution in cerebrospinal-fluid after intravenous, ventricular and lumbar injections. *New Engl. J. Med.* 293 161-166 (1975).
- 5 LUDDY R. E. and GILMAN, P. A. Paraplegia following intrathecal methotrexate. *J. Pediatr.* 83 988-992 (1973).

S. WEISS and Y. HADOT, Roshch Hospital, Tel Aviv (Israel)

The patient died from septicemia, after the reappearance of the leukemia. Permission for autopsy was denied.

Discussion

Paraplegia complicating IT MTX treatment has been reported mainly in children and teenagers and in a few adults [1]. The toxic neurological reaction to MTX therapy can be classified into three groups according to the clinical manifestations [2]: Chemical arachnoiditis, encephalopathy and myelopathy. In myelopathy direct damage to the spinal cord or root nerves may cause serious consequences such as paralysis, transient or permanent paraplegia, quadriplegia and even death. This complication may perhaps be partially preventable.

Different predisposing factors were suggested to be responsible for the development of these neurological complications [1]. These include the chemical preservatives, diluents, and contaminants of MTX. Comparison of 5 patients with severe neurotoxic manifestations post IT MTX with 20 asymptomatic patients receiving the same therapy showed that the toxic patients were significantly older and had overt meningeal leukemia [3]. The same characteristics were found in our patient. Furthermore neurotoxic symptoms occurred in the presence of elevated CSF concentrations of MTX. Therefore, it is suggested that frequent monitoring of CSF MTX concentration may be predictive of serious neurotoxicity and drug dosage and drug interval be adjusted accordingly [3]. However neither the minimal effective concentration of MTX in CSF nor the toxic concentration is known [4] and therefore can not yet serve as a safe guide in treatment or prevention of the neurological complications.

In a recent review of the literature GAOLIANO and COSTANZI [1] found 10 cases of paraplegia following IT MTX therapy and added 1 CSF examinations during IT MTX therapy have been reported in detail in only 2 patients [5].

The elevation of proteins in LUDWIG's case [5] before the onset of paralysis was 33-75-500 mg/dl and in our case 80-60-135 mg/dl. Generally abnormal spinal fluid findings (proteins, cells) respond to IT MTX therapy by decreasing or disappearing. There was no other explanation for the rise of proteins in our case except MTX neurotoxicity as there was no evidence of infection (including TBC), hemorrhage, subarachnoid block (normal myelography) or even arachnoiditis. While there is no one specific reliable indicator of a possible neurotoxic reaction, the discrepancy be

tween proteins and cells may perhaps serve to warn the physician of impending trouble.

It is suggested that a worsening of the neurological condition, or an increase in CSF protein (protein cells dissociation) or pressure during IT MTX therapy be taken as an indication to delay further injection in order to diminish or avoid severe neurological complications.

References

- 1 GAGLIANO, R. B. and COSTANZI, J. J. Paraplegia following intrathecal methotrexate. *Cancer* 37: 1663-1668 (1976).
- 2 DUTTERA, N. J., BLEYER, W. A., POMEROY, T. C., LEVENTHAL, C. M., and LEVENTHAL, B. Irradiation, methotrexate toxicity and the treatment of meningeal leukemia. *Lancet* ii: 703-707 (1973).
- 3 BLEYER, W. A., DRAKE, J. C., and CHAMBER, B. A. Neurotoxicity and elevated cerebrospinal-fluid methotrexate concentration in meningeal leukemia. *New Engl. J. Med.* 289: 770-773 (1973).
- 4 SHAPIRO, W. R., YOCUM, H. F. and MEHTA, B. P. Methotrexate: Distribution in cerebrospinal-fluid after intravenous, ventricular and lumbar injections. *New Engl. J. Med.* 293: 161-166 (1975).
- 5 LUDRY, R. E. and GILMAN, P. A. Paraplegia following intrathecal methotrexate. *J. Pediatr.* 83: 988-992 (1973).

S. WEISS and Y. KADAN, 'Rohach Hospital, Tel Aviv (Israel)

H. H. FUDENBERG (ed.) *Disorders of Lymphopoiesis and Lymphoid Function. Clinica in Haematology* Vol. 6. Saunders, East Sussex 1977 275-339 pp., £ 7.50.

Bewältigung, Wertung und übersichtliche Darstellung der explosiv sich anhäufenden hämatologischen Neuerkenntnisse wird zu einer immer schwierigeren Aufgabe. Es zeichnet sich in den letzten Jahren als sehr verdienstvoll ab, dass die Reihe *Clinica in Haematology* (publiziert bei Saunders), in der dreimal pro Jahr ein 200-300 Seiten starkes Bändchen mit Übersichtsarbeiten zu einem bestimmten hämatologischen Problembereich erscheint, auf diesem Gebiet Hervorragendes leistet. Unter der weislichen Ägide von H. H. FUDENBERG (Charleston) erscheint als Juni Ausgabe 1977 in dieser Reihe ein Bändchen, das in 11 Teilübersichten durch kompetente Fachvertreter über Aufbau und Störungen des lymphatischen Systems berichtet. Gerade bezüglich des Lymphozyten, der zentralen Zelle der Immunologie, ist für den nicht unmittelbar Beteiligten die Übersicht in den letzten Jahren immer schwieriger geworden, was auch die jeweiligen Literaturverzeichnisse der verschiedenen Artikel im zu besprechenden Buche belegen. In dem vorwiegend Arbeiten der 70er Jahre (bis und mit 1976) zitiert werden. Eine aktuelle Standortbestimmung war also dringend und scheint mir auch voll gelungen zu sein.

Im 1. Kapitel werden die zellulären Repräsentanten der Immunantwort vorgestellt (T-Lymphozyten mit ihren funktionellen Untergruppen, B-Lymphozyten, Makrophagen) und auf ihre komplexe zelluläre und humorale Interaktionen eingegangen. Bezeichnenderweise bestehen dann Überschneidungen mit dem 2. Kapitel, wo die Anatomie des Immunsystems zur Darstellung kommt. Hier wird klar, dass es heute nur noch eine funktionelle Anatomie und Morphologie geben kann, morphologisch fixiert wird immer ein bestimmter Funktionszustand einer lymphatischen Zelle oder eines lymphatischen Organes, der im Auge zu behalten ist. Unkorrekt ist die Feststellung auf Seite 300, dass die zytochemische unspezifische Esterase-Reaktion im Lymphozyten negativ sei, gibt es doch jüngstens feste Hinweise, dass die an Mäusen bereits etablierte zytochemische Unterscheidung in B- und T-Zellen auch beim Menschen anwendbar ist. Das 3. Kapitel behandelt die Assoziationen von bestimmten Lymphozyten-Alloantigenen (HLA) mit bestimmten Krankheiten. Im 4. Kapitel wird gründlich auf die Identifikation der mononukleären Zellen aufgrund ihrer Oberflächen-funktionellen und (zyto-)chemischen Marker und deren ontogenetische Differenzierung eingegangen und ihre Anwendung bei Immundefizienz- oder Immunproliferations-Krankheiten aufgezeigt (414 Literatur-Referenzen!). Das 5. Kapitel berichtet über einen neuen Meilenstein in der humanen Immunbiologie: die Definition von Enzymmängeln bei Immunkrankheiten, was ganz neue Aspekte für die Therapie eröffnet (Enzymersatz, Enzymblockierungen). Das 6. Kapitel zeigt die Rolle und Funktion der Suppressor T-Lymphozyten, bezüglich derer sowohl Über- als auch Unterfunktion zu immunologischen Krankheiten führt. Eingebaut in diese Übersicht ist ein interessanter Abschnitt über das Spektrum von der benignen zur malignen Lymphoproliferation. Solche Hinweise auf Regulationsstörungen auch bei Lymphoproliferation weisen auf zukünftige Therapien, die mehr auf Ersatz von fehlenden Regulatoren denn auf Zerstörung von proliferierenden Subpopulationen aus-

gerichtet sein werden. Das 7. Kapitel analysiert Lymphadenopathien und Kollagenosen über dem gemeinsamen Nenner der Dysgammaglobulinämie. Das Buchlein schließt mit einer Übersicht über die normalen und normalen Immunglobulinklassen (Struktur, Synthese, Sekretion).

J. FINK, Zürich

N. N. SEN and A. K. BASU (Hrsg.) *Trends in Haematology* J. B. Chatterjee Memorial Volume. J. B. Chatterjee Memorial Committee, Calcutta 1975 XVI + 506 pp. US \$ 60.00.

Es handelt sich um einen Sammelband zum Gedenken an den im Jahre 1972 verstorbenen indischen Hämatologen, J. B. CHATTERJEE. Der voluminöse Band umfasst 26 Beiträge aus den verschiedensten Gebieten der Hämatologie, abgefasst vom führenden Hämatologen der ganzen Welt. Es waren der Namen wie ROSEMARY BROOKS, CECIL HOUDE, JAN WALDENSTRÖM, D. J. WEATHERALL, ERNEST BEUTLER, G. C. DE GAUCHY V. HEIKERT und G. MATHE wahllos herausgegriffen. Jeder der Beiträge stellt somit eine Übersicht über ein Spezialproblem in hoher Warte der Natürlichkeit, sowohl über die Untersuchungen in Gehalt und Qualität der Präsentation Originalbefunde, soweit überhaupt erwähnt, und meist schon andersorts beschrieben worden. Sicher kann das Buch nicht als eine Einführung in die Hämatologie aufgefasst werden. Es wird aber dem für das Fach in seiner ganzen Breite Interessierten Gelegenheit geben, sich mit Kapiteln, welche nicht seinem eigenen Arbeitsgebiet entsprechen, abzugeben. Leider sind viele Abchnitte schon bei Erscheinen des Buches überholt gewesen, da sich die Publikation offenbar stark hinausgezögert hat. So gehen auch die Literaturhinweise kaum über das Jahr 1972 hinaus. U. BUCHER, Bern

B. S. LEAVELL and O. A. TOROP *Fundamentals of Clinical Haematology* 4th ed. Saunders, Eastbourne 1976 XIII + 736 pp. £ 18.00 ISBN 0-7216-5878-1

The fact that this book appears in its fourth edition shows by itself that it is proved useful. It may be characterized as a concise modern textbook with particular emphasis on the pathophysiological basis of disease. The book follows the conventional order of such reviews. The chapters on disorders of the red cell are preceded by an outline of the origin and morphology of blood cells of erythropoiesis and the metabolism of haemoglobin and are followed by chapters on granulocytes, lymphocytes etc. The book is concluded by its extensive chapters on haemostasis (about 150 pages) and a comparatively short (maybe too short) summary of blood transfusion therapy. Each chapter includes an extensive bibliography. In most chapters well-selected illustrative case histories are presented. This is particularly useful to underline the practical aspects of the theoretical bases. Therapeutic aspects are also carefully dealt with.

On the whole, this book can be recommended, although it is difficult to see who may really profit from reading it. For the average medical student it is certainly too extensive, and the specialist who looks for particular information will probably prefer one of the more extensive texts.

U. BUCHER, Bern

D. A. G. GALTON (Hrsg.) *The Chronic Leukaemias*. Clinics in Haematology vol. 6, No 1 Saunders, Eastbourne 1977 VIII + 274 pp., £ 7.50

Die *Clinics in Haematology* bringen im ersten Bändchen des Jahres 1977 eine von D. A. G. GALTON ausgezeichnet redigierte Übersicht über die chronischen Leukämien in 11 Kapiteln verfasst von kompetenten Autoren aus England, Frankreich, Belgien und den Vereinigten Staaten. Nach einer Besprechung der Epidemiologie der Leukämien unter besonderer Berücksichtigung der chronischen Formen werden die chronisch myelische und die chronisch lymphatische Leukämie getrennt abgehandelt. Neben pathophysiologischen und experimentellen Betrachtungen, insbesondere über zellkinetische Probleme wird besonderes Gewicht auf den klinischen Verlauf und die Symptomatologie sowie die Diagnostik, Differentialdiagnose und Therapie der chronischen Leukosen gelegt. Die neuesten Erkenntnisse über die Zytogenetik der chronischen Myelose sind in einem speziellen Abschnitt übersichtlich und allgemeinverständlich dargelegt. Der Natur der Erkrankung entsprechend stehen bei der chronischen Lymphadenose vor allem die immunologischen Gesichtspunkte sowie die Abgrenzung gegenüber den übrigen lymphoproliferativen Syndromen im Vordergrund. Besondere Beachtung verdient die zusammenfassende Darstellung zweier noch nicht allzu lange bekannter Sonderformen der chronisch lymphatischen Leukämie nämlich der Haarzell Leukämie und der prolymphozytären Leukämie.

Die Lektüre des Bändchens kann allen die sich über den neuesten Stand der Kenntnis über die chronischen Leukämien informieren wollen, bestens empfohlen werden. Die sehr aktuellen Literaturangaben sind ein willkommener Wegleiter zum Studium spezieller Probleme

J. LUDWIG Bern

A. J. COBURN *Die akute normovolämische Hämodilution in klinischer Anwendung*. Anaesthesiology and Resuscitation Vol 104 Berlin, Springer 1977 XI + 89 pp. DM 28 ISBN 3-540-08025-2.

The author presents a careful study on the clinical use of acute normovolemic hemodilution in general surgical patients. An average of 1785 ml of blood was withdrawn prior to surgery and replaced normovolemically by dextran and/or albumin solutions. Despite the fact that these patients were carefully selected in order to exclude higher risk cases, one third displayed moderate to severe hemodynamic side effects and electrocardiographic abnormalities suggesting intraoperative myocardial ischemia. 5 patients collapsed during routine attempts at postoperative mobilization. In the opinion of the author the fact that only 7 patients required homologous blood is a positive result with respect to the prevention of hepatitis. Since, however there is now substantial evidence that the risk of posttransfusion hepatitis has been overrated, because a number of such cases are otherwise acquired during hospitalization, a heavy responsibility continues to rest on the physician who induces acute anemia as a therapeutic measure and thus augments the stress of anaesthesiology and surgery. The monograph is well presented and makes interesting reading for anybody concerned with the dangers of hemodilution.

P. LUNDGAARD-HANSEN Bern

Influence of Food Iron Absorption on the Plasma Iron Level In Idiopathic Hemochromatosis¹

MICHAEL S. MILDER, JAMES E. COOK and CLEMENT A. FINCH

University of Washington, Department of Medicine, Division of Hematology
Seattle, Wash

Key Words. Idiopathic hemochromatosis · Iron absorption · Ferritin

Abstract The relationship between food iron absorption, iron stores, and plasma iron level was studied. On a low iron diet subjects with idiopathic hemochromatosis (IH) during reaccumulation of iron after phlebotomies showed a fall in plasma iron. Fortification of the diet with 22.5 mg of iron/day for 3 days caused little or no change in the plasma iron in subjects with normal iron stores, whereas in subjects with iron deficiency a significant rise in plasma iron occurred with the addition of 45 mg of iron/day. In subjects with IH with normal iron stores, plasma iron increased with the addition of 22.5 mg/day. These studies indicate that iron absorption is an important determinant of the elevated plasma iron in IH and that the plasma iron tolerance test combined with the serum ferritin may be used to detect excessive absorption of iron.

Introduction

Idiopathic hemochromatosis is a genetic disorder in which there is increased iron absorption leading to parenchymal iron overload [21]. The reticuloendothelial system and the intestinal mucosa appear to share an inability to retain iron [9-17]; as a result, iron from red cell catabolism

This work was supported in part by National Institutes of Health grants HL 00644 and AM-05130. A portion of this work was conducted through the Clinical Research Center facility of the University of Washington supported by the National Institutes of Health (grant RR 37).

along with iron taken up by the mucosa from the intestinal lumen is transferred to the plasma. This leads to overload of the plasma transferrin compartment, and the only storage area available for this excess iron is the hepatocyte and other parenchymal tissues. Previous studies have shown that iron absorption in idiopathic hemochromatosis is abnormally high relative to the amount of body iron stores [15-21]. In subjects with marked iron overload, absorption of nonheme food iron is equal to or greater than that of normal subjects. After the excessive stores have been depleted, the absorption of nonheme iron equals or exceeds levels seen in iron deficiency.

Idiopathic hemochromatosis is usually diagnosed on the basis of characteristic signs and symptoms and laboratory tests indicating iron overload including transferrin saturation, serum ferritin, desferrioxamine-induced urinary iron excretion, and ultimately liver biopsy [18]. However, in the early stages of the disease clinical manifestations are absent and even the usual laboratory findings may not be abnormal. At such a time increased iron absorption may be the only clue to the underlying disorder [1-20]. The purpose of this study is to demonstrate the relationship between iron absorption, plasma iron concentration, and serum ferritin concentration, and to utilize these relationships to detect excessive absorption. Two situations may be encountered in idiopathic hemochromatosis in the early stage of iron loading: (1) individuals with elevated plasma iron but not greatly enlarged iron stores, in whom the role of absorption in maintaining the iron level may be examined by placing the patients on a low iron diet; and (2) subjects with a normal plasma iron, in whom excessive food iron absorption may be detected by changes in the plasma iron concentration.

Methods

Iron Deprivation Test

Three groups of patients were studied: (1) iron replete normal volunteers, (2) subjects with untreated advanced idiopathic hemochromatosis, and (3) subjects with hemochromatosis in whom the plasma iron had been brought down to normal by phlebotomies and then allowed to return to a high level. Data on the hematologic and iron status of these subjects is summarized in table I. The diagnosis of hemochromatosis was based on elevated transferrin saturation and ferritin levels, a family history of hemochromatosis, and marked parenchymal iron overload on liver biopsy.

The subjects were followed for 7 days on an unrestricted regular diet. They were then given a low iron diet for 7 days followed by a return to their normal diet. The

Table I Data on subjects in iron deprivation study

	Normal	Untreated hemochromatosis	Treated hemochromatosis
Number	2	2	3
Hematocrit, %	46-48	39-49	41.5-48
Plasma iron, $\mu\text{g/dl}$	125-159	234-267	204-294
Transferrin saturation, %	41-43	92-95	79-98
Ferritin, $\mu\text{g/l}$	55-72	1 770-3 400	23-470

Table II Data on subjects in iron supplementation study

	Normal	Iron depleted	Idiopathic hemochromatosis
Number	20	5	6
Male/female	7/13	1/4	5/1
Hematocrit, %			
Mean	41.0	37.2	40.3
Range	37-47	33-42	37-43
Ferritin, $\mu\text{g/l}$			
Mean	71.2	8.2	85.4
Range	20-220	1-14	19-290
Transferrin saturation, %			
Mean	32	10	34
Range	15-56	6-18	17-52
Plasma iron, $\mu\text{g\%}$			
Mean	107.1	39.2	101.8
Range	54-188	21-65	49-139

low iron diet consisted of infant formula (Elsamal without iron, Mead Johnson) diluted with water and containing 2 mg of iron/l. A average daily iron intake on this formula ranged from 2.3 to 4.1 mg in different subjects. Plasma iron was determined at 12-hour intervals during the study and iron-binding capacity was measured at weekly intervals.

Food Iron Fortification Test

Three groups of subjects were studied (1) iron replete normal volunteers, (2) volunteers with iron depletion but with little or no anemia, and (3) subjects with idiopathic hemochromatosis in whom the plasma iron and iron stores had been reduced to normal by phlebotomy. Hematologic and iron data on these subjects are

summarized in table II. Normal subjects had transferrin saturations of 16% or greater on a midday blood sample, average ferritin of 20 ng/ml or greater and red cell protoporphyrin of $< 50 \mu\text{g/dl}$. The 5 subjects with iron deficiency all had serum ferritin levels of $< 15 \text{ ng/ml}$. 4 had a transferrin saturation of $< 13\%$, while the fifth had a saturation of 17% but absent iron stores on bone marrow examination. The criteria for diagnosis of hemochromatosis were the same as stated above. In 1 patient a liver biopsy was not performed, but the patient had a family history of the disease and an elevated plasma iron and ferritin.

All subjects took their usual diet during the 7-day study. On days 3 through 5 a solution of ferrous sulfate was swallowed during each of three meals in doses ranging from 7.5 to 45 mg/meal. Plasma iron was measured each day $\sim 3 \text{ h}$ after the evening meal and at other times in selected studies. The evening sample was selected to maximize the difference between the normal and abnormal response and to take advantage of the relative stability of the evening serum iron level in comparison with the morning level. Total plasma iron-binding capacity, serum ferritin, and hematocrit were measured at the beginning and end of the study. Plasma iron was measured by the Iron Panel method [1] and total iron binding capacity by the magnesium carbonate method [4]. Plasma ferritin levels were measured by immunoradiometric assay [14].

Results

Iron Deprivation Test

The results of the iron deprivation test are shown in figure 1. In the 2 normal subjects plasma iron ranged between 80 and $170 \mu\text{g/dl}$. No significant change was observed during the period of iron deprivation with both maintaining a level between 75 and $130 \mu\text{g/dl}$. In patients with untreated idiopathic hemochromatosis the plasma iron remained above $200 \mu\text{g/dl}$ with transferrin saturation exceeding 90% during the entire study. Four studies were done in 3 subjects with hemochromatosis after phlebotomy had returned their iron stores to normal. All had consistently high plasma iron concentrations despite large fluctuations during the control period. The plasma iron fell a mean of $118.4 \mu\text{g/dl}$ (range 93–163 $\mu\text{g/dl}$) 3–6 days after the institution of the low iron diet and the decrease persisted until about 4 days after a normal diet had been resumed.

Food Iron Fortification Study

29 trials on 20 normal subjects were carried out using doses of 7.5, 15, 22.5, 30, and 45 mg of added iron/meal. Allowing for an average dietary iron content of 15 mg/day, these subjects had a total daily intake of about 35, 60, 80, 105, and 150 mg of iron. The average plasma iron response at each dose level is shown in figure 2. Over the entire dose range

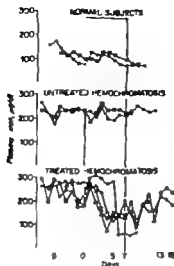


Fig 1 Daily plasma iron concentration in three groups of patients taking low-iron diet on day 1 through 7 (shaded). Patients with treated hemochromatosis had been phlebotomized to normal iron stores.



Fig 2 Daily plasma iron (mean \pm SE) of normal subjects with food iron fortification on days 3, 4 and 5 (FE). The number of subjects in each group is 5: 7.5 mg/meal, 6 at 13 mg/meal, 6 at 22.5 mg/meal, 7 at 30 mg/meal, and 5 at 45 mg/meal.

there was no appreciable change in the evening plasma iron concentration. For normal subjects at all doses, the plasma iron on the first day of iron fortification (day 3) averaged $13.3 \mu\text{g/dl}$ higher than during the control period, $7.5 \mu\text{g/dl}$ higher on the second day (day 4) and $1.7 \mu\text{g/dl}$ lower on the third day (day 5). Transient increments as great as $92 \mu\text{g/dl}$ were seen on days 3 and 4 at the two higher dose levels. The largest increment on day 5 at any dose was $40 \mu\text{g/dl}$.

With 7.5 mg of added iron meal ($\sim 5 \text{ mg/day}$) none of 5 subjects

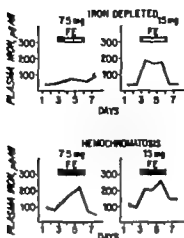


Fig 3 Daily plasma iron (mean \pm SE) in iron fortification trials of patients with iron depletion and idiopathic hemochromatosis after phlebotomy to normal iron stores.

showed an increase $> 15 \mu\text{g/dl}$. The average change was $+13.7 - 5.7$ and -15.1 on days 3, 4, and 5, respectively. On 15 mg of added iron/meal (45 mg/day) the largest increase was $61 \mu\text{g/dl}$ with an average of $+7.9 + 2.7$ and -0.4 on days 3, 4, and 5, respectively.

In the iron-depleted subjects with little or no anemia, there was little difference from the normal response in four studies using 7.5 mg of added iron/meal. Plasma iron changes of -1.1 , $+19.6$, and $+9.6$ were observed on days 3, 4, and 5, respectively. However, 4 subjects on a dose of 15 mg/meal and the fifth on 30 mg/meal showed marked increases in plasma iron. Average increments on days 3, 4, and 5 were 156.7 , 122.1 , and $118.7 \mu\text{g/dl}$, respectively. 4 of the 5 had increments over $100 \mu\text{g/dl}$ on day 3, 3 on day 4, and 2 on day 5. All were within $35 \mu\text{g/dl}$ of the control level by day 6.

Patients with idiopathic hemochromatosis but with a normal transferrin saturation showed an even greater response to food iron fortification (fig. 3). In 4 patients given 7.5 mg of iron/meal, 1 had an increment of over $150 \mu\text{g/dl}$ on day 3, 2 on day 4, and 3 on day 5. The mean increases were 43.2 , 93 , and $130 \mu\text{g/dl}$. When 15 mg of iron/meal was given to 6 subjects, all showed prompt and sustained rises in plasma iron with five reaching transferrin saturations of above 90%. Mean increments were 102.6 on day 3, 106.8 on day 4, and 158.2 on day 5. Increases ranged from 133 to $234 \mu\text{g/dl}$ on day 5.

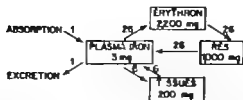


Fig 4 Model of iron exchange in man. Average size of each compartment is shown in rectangles for adult male. Daily iron exchange between compartments is shown in milligrams on arrows.

Discussion

The iron supply available to body tissues at any given time is reflected in the plasma iron level [10]. This compartment usually contains only 2–4 mg of iron, but enlarges two- to threefold in parenchymal loading states. Because the plasma iron compartment is small relative to total iron turnover it can be a sensitive indicator of alterations in input, provided erythropoiesis remains stable and diurnal changes are considered.

Most of the iron entering the plasma is derived from processing of red cells by the reticuloendothelial system and is counterbalanced by an equivalent outflow to the erythron to replace the red cells lost (fig. 4). A smaller cycle of iron enters extravascular fluids, returning through the lymphatics [10]. The only real gain in the system is the iron entering the gut, and this is so regulated as to replace body iron losses of about 1 mg/day [2]. This is accomplished by absorption of 5–10% of dietary iron [2]. Ordinarily the plasma iron concentration in man is not affected in any recognizable way by the daily absorption of 1 mg of iron from meals; indeed, the plasma iron usually falls during the day when most of this absorption is occurring. These studies have clearly shown that far greater amounts of iron ingested with food may have no recognizable effect on the plasma iron in subjects with normal iron stores. On the other hand, increased absorption, whether diagnosed as iron deficiency or idiopathic hemochromatosis, is reflected in an increase in plasma iron after ingestion of an iron fortified diet.

Iron absorption is closely regulated in the normal individual, the most important determinant of absorption being the amount of iron stores [3, 16]. With iron store depletion absorption is increased, and with increased stores absorption decreases. It is therefore critical in determining the appropriateness of iron absorption to evaluate it in the context of body iron

stores. These may be evaluated by the plasma ferritin level [7-19]. Mean values among adult females are about 35 $\mu\text{g/l}$ and among males 100 $\mu\text{g/l}$ corresponding to iron stores of about 300 and 900 mg respectively [7-19]. It has been shown that iron absorption is inversely correlated with the ferritin level [7].

Perhaps the best situation in which to test the effect of absorbed iron on serum iron concentration is the patient with idiopathic hemochromatosis whose iron stores have been reduced to normal. Absorption in such individuals is excessive [21] and plasma iron is usually high. The interdependence of the two was shown by studies reducing the iron content of the diet to about 20% of normal. There was a 3-day lag before the plasma iron concentration fell indicating the importance of looking for sustained changes in plasma iron rather than rapid fluctuations. Similar reductions in plasma iron are seen in rats when available dietary iron is reduced [6]. At the same time it was shown that patients with greatly enlarged iron stores maintain an elevated plasma iron independent of dietary iron absorption due to large amounts of iron entering the plasma from storage pools.

Further studies were undertaken to examine absorption of iron by normoferremic individuals. Since abnormalities in absorption may relate to luminal as well as mucosal factors, and since iron overload develops as a result of excessive food iron absorption dietary iron intake was augmented by the addition of ferrous sulfate. It is known that added iron salts mix with nonheme food iron pool and are subject to the same inhibiting and facilitating factors as is natural food iron [5]. This applies to the addition of amounts of iron of the magnitude employed in this study [13].

The general relationship between iron stores as evaluated by ferritin, amount of iron absorbed, and the plasma iron elevation have been previously described [7-8]. In this study these relationships are extended to food iron. The use of food iron results in much lower absorption so that iron doses of as much as 45 mg/meal in normal subjects gave little or no response whereas plasma iron increases occurred with fortification by 15 mg/meal in iron depleted subjects and 7 mg in idiopathic hemochromatosis. The study of absorption over several days allows identification of differing patterns of response. Normal and iron-depleted subjects appear to decrease absorption with repeated doses [11]. In contrast patients with hemochromatosis increase their iron response. Thus, after 3 days of iron fortification the hemochromatosis group is most clearly separated from those with normal regulation of absorption.

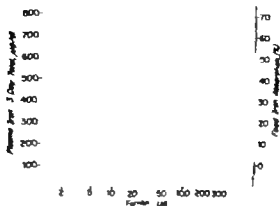


Fig 5 Relationship between the serum ferritin (μ grams) and the sum of plasma iron levels during three days of food iron fortification (left ordinate) normal (O), iron depleted (\square), and treated hemochromatotic (Δ) subjects. The shaded area indicates the expected relationship normal subjects between iron absorption (right ordinate) and ferritin (on abscissa) as derived from the data of Cook *et al* [7]

These results suggest that the relationship of the plasma iron response and iron stores as measured by ferritin can differentiate physiologic iron absorption from pathologic absorption seen in hemochromatosis. In figure 5 the plasma iron levels with fortified diet are plotted against the ferritin level. Absorption of radioiron taken with a standard meal follows the same pattern in subjects with normal regulation of absorption by iron stores [7]. The serum iron response in hemochromatosis falls well off this curve showing increased plasma iron response at every level of iron stores studied.

In summary iron absorption is a major determinant of the plasma iron level in subjects with idiopathic hemochromatosis in an early stage of iron loading. In normal subjects, absorbed iron does not influence the plasma level unless iron stores are depleted or intake is greatly augmented (greater than 150 mg/day).

References

- 1 BAUCHERAE, S. P., WILKINSON, M. H., LE, R. F. and DOYLE, A. P. Idiopathic hemochromatosis. A study of three families. *Am J Med* 42: 857-873 (1966)

stores. These may be evaluated by the plasma ferritin level [7-19]. Mean values among adult females are about 35 $\mu\text{g/l}$ and among males 100 $\mu\text{g/l}$, corresponding to iron stores of about 300 and 900 mg respectively [7-19]. It has been shown that iron absorption is inversely correlated with the ferritin level [7].

Perhaps the best situation in which to test the effect of absorbed iron on serum iron concentration is the patient with idiopathic hemochromatosis whose iron stores have been reduced to normal. Absorption in such individuals is excessive [21] and plasma iron is usually high. The interdependence of the two was shown by studies reducing the iron content of the diet to about 20% of normal. There was a 3-day lag before the plasma iron concentration fell, indicating the importance of looking for sustained changes in plasma iron rather than rapid fluctuations. Similar reductions in plasma iron are seen in rats when available dietary iron is reduced [6]. At the same time it was shown that patients with greatly enlarged iron stores maintain an elevated plasma iron independent of dietary iron absorption due to large amounts of iron entering the plasma from storage pools.

Further studies were undertaken to examine absorption of iron by non-ferremic individuals. Since abnormalities in absorption may relate to luminal as well as mucosal factors, and since iron overload develops as a result of excessive food iron absorption, dietary iron intake was augmented by the addition of ferrous sulfate. It is known that added iron salts mix with nonheme food iron pool and are subject to the same inhibiting and facilitating factors as is natural food iron [5]. This applies to the addition of amounts of iron of the magnitude employed in this study [13].

The general relationship between iron stores as evaluated by ferritin, amount of iron absorbed and the plasma iron elevation have been previously described [7-8]. In this study these relationships are extended to food iron. The use of food iron results in much lower absorption so that iron doses of as much as 45 mg/meal in normal subjects gave little or no response whereas plasma iron increases occurred with fortification by 15 mg/meal in iron depleted subjects and 7 mg in idiopathic hemochromatosis. The study of absorption over several days allows identification of differing patterns of response. Normal and iron-depleted subjects appear to decrease absorption with repeated doses [11]. In contrast, patients with hemochromatosis increase their iron response. Thus, after 3 days of iron fortification the hemochromatosis group is most clearly separated from those with normal regulation of absorption.

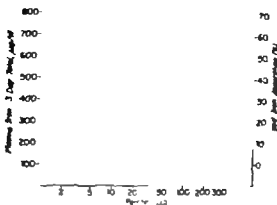


Fig 5 Relationship between the serum ferritin (abscissa) and the size of plasma iron level during three days of food iron fortification (left ordinate) in normal (\circ), iron depleted (\square), and treated hemochromatotic (\triangle) subjects. The shaded area indicates the expected relationship in normal subjects between iron absorption (right ordinate) and ferritin (on abscissa) as derived from the data of COOK *et al* [7].

These results suggest that the relationship of the plasma iron response and iron stores as measured by ferritin can differentiate physiologic iron absorption from pathologic absorption seen in hemochromatosis. In figure 5 the plasma iron levels with fortified diet are plotted against the ferritin level. Absorption of radioiron taken with a standard meal follows the same pattern in subjects with normal regulation of absorption by iron stores [7]. The serum iron response in hemochromatosis falls well off this curve, showing increased plasma iron response at every level of iron stores studied.

In summary iron absorption is a major determinant of the plasma iron level in subjects with idiopathic hemochromatosis at an early stage of iron loading. In normal subjects, absorbed iron does not influence the plasma level unless iron stores are depleted or intake is greatly augmented (greater than 150 mg/day).

References

- 1 BALCITZAK, S. D., WYTHEMAN, M. P., LE, R. F. and DODS, A. F. Idiopathic hemochromatosis: A study of three families. *Am J Med* 42: 857-873 (1966).

stores. These may be evaluated by the plasma ferritin level [7-19]. Mean values among adult females are about 35 $\mu\text{g/l}$ and among males 100 $\mu\text{g/l}$, corresponding to iron stores of about 300 and 900 mg respectively [7-19]. It has been shown that iron absorption is inversely correlated with the ferritin level [7].

Perhaps the best situation in which to test the effect of absorbed iron on serum iron concentration is the patient with idiopathic hemochromatosis whose iron stores have been reduced to normal. Absorption in such individuals is excessive [21] and plasma iron is usually high. The interdependence of the two was shown by studies reducing the iron content of the diet to about 20% of normal. There was a 3-day lag before the plasma iron concentration fell, indicating the importance of looking for sustained changes in plasma iron rather than rapid fluctuations. Similar reductions in plasma iron are seen in rats when available dietary iron is reduced [6]. At the same time it was shown that patients with greatly enlarged iron stores maintain an elevated plasma iron independent of dietary iron absorption due to large amounts of iron entering the plasma from storage pools.

Further studies were undertaken to examine absorption of iron by normal ferremic individuals. Since abnormalities in absorption may relate to luminal as well as mucosal factors, and since iron overload develops as a result of excessive food iron absorption, dietary iron intake was augmented by the addition of ferrous sulfate. It is known that added iron salts mix with nonheme food iron pool and are subject to the same inhibiting and facilitating factors as is natural food iron [5]. This applies to the addition of amounts of iron of the magnitude employed in this study [13].

The general relationship between iron stores as evaluated by ferritin, amount of iron absorbed, and the plasma iron elevation have been previously described [7-8]. In this study these relationships are extended to food iron. The use of food iron results in much lower absorption so that iron doses of as much as 45 mg/meal in normal subjects gave little or no response whereas plasma iron increases occurred with fortification by 15 mg/meal in iron depleted subjects and 7 mg in idiopathic hemochromatosis. The study of absorption over several days allows identification of differing patterns of response. Normal and iron-depleted subjects appear to decrease absorption with repeated doses [11]. In contrast, patients with hemochromatosis increase their iron response. Thus, after 3 days of iron fortification the hemochromatosis group is most clearly separated from those with normal regulation of absorption.

- 19 WALTERS, G. O., MILLER, F. M. and WORWOOD, III. Serum ferritin concentration and iron stores in normal subjects. *J. clin. Path.* 26: 770-772 (1973).
- 20 WILLIAMS, R., PRITCHER, C. S., PARSONSON, A., and WILLIAMS, H. S. Iron absorption in the relatives of patients with idiopathic hemochromatosis. *Lancet* *i*: 1243-1246 (1965).
- 21 WILLIAMS, R., MAIENTI, F., WILLIAMS, H. S. and PRITCHER, C. S. Iron absorption in idiopathic hemochromatosis before, during, and after venesection therapy. *Br. med. J.* *A*: 78-81 (1966).

- 2 BOTHWELL, T. H. and FINCH, C. A., Iron metabolism 1st ed. (Little Brown, Boston 1962)
- 3 CONRAD M. E., JR and CROSBY, W. H., Intestinal mucosal mechanisms controlling iron absorption. *Blood* 22 406-415 (1963).
- 4 COOK, J. D. An evaluation of absorption methods for measurement of plasma iron-binding capacity. *J Lab clin Med* 76 497-506 (1970)
- 5 COOK, J. D., LAYRISSE, M., MARTINEZ TORRES, C., WALKER, R. and FINCH, C. A. Food iron absorption measured by an extrinsic tag. *J clin. Invest* 51 805-815 (1972).
- 6 COOK, J. D. HERSHKO, C., and FINCH, C. A. Storage iron kinetics. V Iron exchange in the rat. *Br J Haemat.* 25 695-706 (1973)
- 7 COOK, J. D. LIPSCHITZ, D. A. MILES, L. E. M. and FINCH, C. A., Serum ferritin as a measure of iron stores in normal subjects. *Am J clin. Nutr* 27 681-687 (1974).
- 8 EKENVED, G. Iron absorption studies. Studies on oral iron preparations using serum iron and different radioiron isotope techniques. *Scand. J. Haematol suppl* 28, pp 7-97 (1976).
- 9 FILLET G and MARRAGLIA, G. Idiopathic hemochromatosis (IH) Abnormality in RBC transport of iron by the reticuloendothelial system (RES). *Abstract Blood* 46. 1007 (1975)
- 10 FINCH C. A. DEUELBEKER, K. COOK, J. D. ESCHBACH, J. W. HARKER, L. A., FUNK, D. D. MARRAGLIA, G. HILLMAN R. S. SLICHTER S. ADAMSON J. W. GANZONI, A., and GIBLETT E. R. Ferrokinetics in man. *Medicine* 49 17-53 (1970)
- 11 HODLUND, S. Studies in iron absorption. VI Transitory effect of oral administration of iron on iron absorption. *Blood* 34 505-510 (1969).
- 12 International Committee for Standardization in Hematology. Proposed recommendations for measurement of serum iron in human blood. *Blood* 37 598-600 (1971)
- 13 LAYRISSE, M. and MARTINEZ TORRES, C., Food iron absorption. Iron supplementation of food. *Prog Hematol* 7 137-160 (1971)
- 14 MILES, L. E. M. LIPSCHITZ, D. A. BIEBER, C. P. and COOK, J. D. Measurement of serum ferritin by a β -site immunoradiometric assay. *Analyt. Biochem.* 61 209-224 (1974)
- 15 PIRZIO-BIROLI G. BOTHWELL, T. H. and FINCH, C. A. Iron absorption. II The absorption of radioiron administered with a standard meal in man. *J Lab clin. Med.* 51 37-48 (1958)
- 16 PIRZIO-BIROLI G. and FINCH C. A. Iron absorption III The influence of iron stores on iron absorption in the normal subject. *J Lab clin Med.* 53 216-220 (1960)
- 17 POWELL, L. S. CAMPBELL, C. B., and WILSON E. Intestinal mucosal uptake of iron and iron retention in idiopathic hemochromatosis: a evidence for a mucosal abnormality. *Gut* 11 727-731 (1970)
- 18 WALKER R. J. and WILLIAMS, R. Haemochromatosis and iron overload. Iron in biochemistry and medicine (Academic Press, London 1974)

- 19 WALTERS, D. D. MILLER, F. M., and WOODWARD, M. Serum ferritin concentration and iron stores in normal subjects. *J. clin. Path.* 26: 770-772 (1973).
- 20 WILLIAMS, R., FITCHER, C. S., PARSONSON, A., and WILLIAMS, H. S. Iron absorption in the relatives of patients with idiopathic hemochromatosis. *Lancet* *i*: 1243-1246 (1965).
- 21 WILLIAMS, R., MANENTI, F. WILLIAMS, H. S., and FITCHER, C. S. Iron absorption in idiopathic hemochromatosis before, during, and after venesection therapy. *Br. med. J. d.* 78-81 (1966).

Decreased Iron and Zinc Absorption in Turkish Children with Iron Deficiency and Geophagia¹

AYTEN ARCASOY AYHAN O ÇAYDAR and EMEL BABACAN

Hematology Division of the Pediatric Department of the
Medical School of Ankara University Ankara

Key Words Geophagia Iron absorption Zinc absorption Duodenal Biopsy

Abstract Oral iron and zinc tolerance tests were performed in 12 patients between 8 and 21 years of age, with iron deficiency anemia and geophagia. Decreased iron and zinc absorption were detected respectively in patients against the elevated absorption curves in control subjects. Iron and zinc malabsorption may be an additional feature of the syndrome characterized by geophagia, iron deficiency anemia, hepatosplenomegaly hypogonadism and dwarfism observed in Turkey and Iran.

Iron deficiency is the most frequent cause of anemia throughout the world [12]. Nutritional factors, especially inadequate diet and geophagia, are the main causes of iron deficiency among children in Turkey [4, 10]. Iron deficiency has been considered as one of the public health problems in our country.

Anemia is one of the several systemic manifestations of iron deficiency [12]. The structural and functional changes in the small intestine have been observed in patients with iron deficiency anemia by several authors [1, 5, 7, 8, 11].

It is generally known that iron absorption is increased in iron deficient subjects, although impaired absorption of d xylose, fat and vitamin A may be present [5, 7, 11]. It is the purpose of this article to report de

This study has been supported by Turkish Scientific and Technical Research Council (TBTAK).

Received March 30 1977 accepted November 18, 1977

creased iron and zinc absorption in patients with iron deficiency anemia and geophagia.

Material and Method

12 village children and adolescents with prolonged geophagia and anemia were studied. The subjects were detected during our geophagia survey while iron and zinc absorption tests were routinely carried out during the recent years. Their ages ranged from 8 to 21 years and 6 of them were girls. They were hospitalized during the time that they were studied. In addition, 5 children with iron deficiency anemia but without geophagia, served as controls.

Routine hematological tests were performed through the standard technique. Serum iron and total iron binding capacity (TIBC) were measured according to previously described methods [2, 4].

The diagnosis of iron deficiency anemia are established in all cases by determining the serum iron, total iron-binding capacity and transferrin saturation level of $< 16\%$, in the presence of hypochromic, microcytic anemia.

Iron absorption was measured through the oral plasma iron tolerance test as was described previously [2, 4]. A test dose of 65 mg elemental iron in the form of ferrous fumarate was given orally to the fasting children. Blood samples were obtained for serum iron analysis at 2, 4 and 6 h following the test dose of iron.

Furthermore, zinc absorption was measured in 5 patients and 5 healthy control subjects through the plasma zinc tolerance test that was developed. Following the

Table 1 Clinical findings in the patients

Case No	Age, years	Sex	Duration of geophagia years	Growth retardation	Centimeters below costal margin		Bone age, years
					liver	spleen	
1	E.B.	9	F	8	3	7	3
	L.K.	9	F		3	6	6
3	F.T.	13	F	4	3	N	8-9
4	G.C.	16	F	6	3	1	N
5	R.T.	15	M	5	3	N	
6	A.E.	8	M	5	3	N	
7	H.K.	17	M	15	3	6	10
8	S.T.	11	F	5	1	4	13
9	O.A.	14	M	1	10	5.5	7.5
10	C.A.	1	M	5	3	N	Palpable
11	A.K.	18	M	6	3	6	8
12	G.G.	11	F	8	3	2.5	10

- Not studied N = normal

Geophagia is not continuing

Table II Hematological findings in the patients and controls

Case No	Age years	Sex	Hemo- globin g/dl	Hemato- crit %	Serum iron $\mu\text{g/dl}$ (97.9 \pm 27) ¹	TIBC $\mu\text{g/dl}$ (300-350) ¹	TS, %	Serum zinc $\mu\text{g/dl}$ (105.8 \pm 4.5) ¹
<i>Patients</i>								
1 E.B	9	F	5.0	-	54	381	14.1	80
2 L.K	9	F	5.1	20	4	384	6.2	98
3 F.T	13	F	5.7	20	48	723	6.6	-
4 G.Ç	16	F	5.9	19	56	506	11.0	-
5 R.T	15	M	6.1	20	38	488	7.7	80
6 A.E.	8	M	7.1	-	43	493	8.7	-
7 H.K.	17	M	5.2	21	44	764	5.8	75
8 S.T	11	F	5.8	23	20	410	4.8	70
9 O.A.	14	M	6.1	-	28	448	6.2	80
10 C.A	21	M	4.7	19	56	351	10.1	72.0
11 A.K.	14	M	3.6	-	60	660	9.1	80.0
12 G.G	11	F	5.0	-	60	654	9.3	80
<i>Control</i>								
1 S.G			6.2				6.3	
2 B.Y			7.0				5.5	
3 M.U			6.3				7.7	
4 H.S			6.2				7.2	
5 I.Ç			6.1				13.0	

TIBC = Total iron-binding capacity TS = transferrin saturation.

¹ Normal mean values and SD

² Normal value.

collection $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ containing 27 mg elemental zinc was administered orally to the subjects. Blood samples were obtained at 0, 4 and 6 h following the test dose of zinc. The absorption curves were obtained in the similar way to iron curves. The serum zinc levels were measured by atomic absorption spectrophotometer Perkin-Elmer model 103 [13]

Finally peroral intestinal biopsy was performed in 4 patients and histopathological examinations were obtained.

Results

Clinical and hematological findings have been shown in tables I and



Fig 1 2 9-year-old girl with thalassemia-like appearance.

The duration of geophagia in the form of clay eating ranged from 4 to 15 years. All patients but one had severe growth retardation (below the 3rd percentile in height) and 7 showed hypogonadism.

Of the 12 cases, 9 had splenomegaly (from palpable spleen to 13 cm below the left margin) and 8 had hepatomegaly. It was interesting to note that 3 patients had facial appearances resembling *thalassaemia major* as well as hair-on-end appearance on skull X ray as shown in figures 1-3. However, hematologic studies including A₂ and F determination were not compatible with the diagnosis of thalassemia. Bone age was retarded in 6 patients out of 9 who had roentgenological studies. Table II presents the pertinent hematologic data. They all had severe anemia, as initial hemoglobin level ranged from 3.6 to 7 g/dl. Serum iron levels were decreased

Table II Hematological findings in the patients and controls

Case No.	Age years	Sex	Hemo- globin g/dl	Hemato- crit %	Serum iron $\mu\text{g/dl}$ (97.9 \pm 27) ¹	TIBC $\mu\text{g/dl}$ (300-350) ¹	TS, %	Serum zinc $\mu\text{g/dl}$ (105.6 \pm 22.5) ¹	
<i>Patients</i>									
1	E.B	9	F	5.0	—	54	381	14.1	80
2	L.K	9	F	5.1	20	24	384	6.2	98
3	F.T	13	F	5.7	20	48	723	6.6	—
4	G.Ç	16	F	5.9	19	56	506	11.0	—
5	R.T	15	M	6.2	20	38	488	7.7	80
6	A.E.	8	M	7.2	—	43	493	8.7	—
7	H.K	17	M	5.2	21	44	764	5.8	75
8	S.T	11	F	5.8	23	20	410	4.8	70
9	O.A	14	M	6.1	—	28	448	6.2	80
10	C.A.	21	M	4.7	19	56	551	10.1	72.0
11	A.K	14	M	3.6	—	60	660	9.2	80.0
12	G.G	11	F	5.0	—	60	654	9.3	80
<i>Control</i>									
1	S.G			6.2				6.3	
2	B.Y			7.0				5.5	
3	M.U			6.3				7.7	
4	H.S			6.2				7.2	
5	I.Ç.			6.1				13.0	

TIBC = Total iron-binding capacity TS = transferrin saturation

Normal mean values and SD

¹ Normal value.

collection $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ containing 27 mg elemental zinc was administered orally to the subjects. Blood samples were obtained at 2, 4 and 8 h following the test dose of zinc. The absorption curves were obtained in the similar way to iron curves. The serum zinc levels were measured by atomic absorption spectrophotometer Perkin-Elmer model 103 [13].

Finally peroral intestinal biopsy was performed in 4 patients and histopathological examinations were obtained.

Results

Clinical and hematological findings have been shown in tables I and



Fig 1 2 9-year-old girl with thalassemia-like appearance.

The duration of geophagia in the form of clay eating ranged from 4 to 15 years. All patients but one had severe growth retardation (below the 3rd percentile in height) and 7 showed hypogonadism.

Of the 12 cases, 9 had splenomegaly (from palpable spleen to 13 cm below the left margin) and 8 had hepatomegaly. It was interesting to note that 3 patients had facial appearances resembling to thalassemia major as well as 'hair-on-end' appearance on skull X ray as shown in figures 1-3. However hematologic studies including A_2 and F determination were not compatible with the diagnosis of thalassemia. Bone age was retarded in 6 patients out of 9 who had roentgenological studies. Table II presents the pertinent hematologic data. They all had severe anemia, as initial hemoglobin level ranged from 3.6 to 7.2 g/dl. Serum iron levels were decreased

Table 11 Hematological findings in the patients and controls

Case No.	Age years	Sex	Hemo- globin g/dl	Hemato- crit %	Serum iron µg/dl (97.9 ± 27) ¹	TIBC µg/dl (300-350) ¹	TS, %	Serum zinc µg/dl (105 ± 2.5) ¹
<i>Patients</i>								
1 E.B	9	F	5.0	-	54	331	14.1	80
2 L.K	9	F	5.1	20	24	384	6.2	98
3 F.T	13	F	5.7	20	48	723	6.6	-
4 G.Ç	16	F	5.9	19	56	506	11.0	-
5 R.T	15	M	6.2	20	38	488	7.7	80
6 A.E.	8	M	7.2	-	43	493	8.7	-
7 H.K	17	M	5.2	21	44	764	5.8	75
8 S.T	11	F	5.8	23	20	410	4.8	70
9 O.A	14	M	6.1	-	28	448	6.2	80
10 C.A.	21	M	4.7	19	56	551	10.1	72.0
11 A.K	14	M	3.6	-	60	660	9.2	80.0
12 G.G	11	F	5.0	-	60	654	9.3	80
<i>Control</i>								
1 S.G			6.2				6.3	
B.Y			7.0				5.5	
3 M.U			6.3				7.7	
4 H.S.			6.2				7.2	
5 I.Ç			6.1				13.0	

TIBC = Total iron-binding capacity TS = transferrin saturation.

Normal mean values and SD

¹ Normal value.

collection ZnSO₄ · 7H₂O containing 27 mg elemental zinc was administered orally to the subjects. Blood samples were obtained at 2, 4 and 6 h following the test dose of zinc. The absorption curves were obtained in the similar way to iron curves. The serum zinc levels were measured by atomic absorption spectrophotometer Perkin-Elmer model 103 [13].

Finally peroral intestinal biopsy was performed in 4 patients and histopathological examinations were obtained.

Results

Clinical and hematological findings have been shown in tables I and

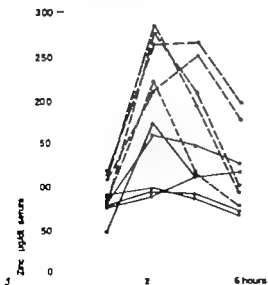
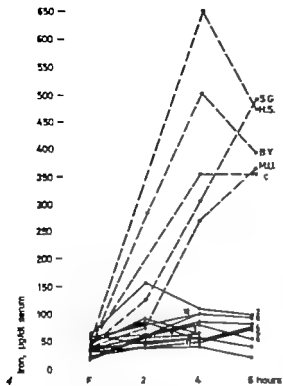




Fig 3 Skull X ray of the same patient. Note the 'hair-on-end' appearance of the parietal bones.

and TIBC increased in all cases. Transferrin saturation was below 10% in 9 patients indicating severe iron deficiency anemia.

The children used as controls in this study showed hematological findings similar to those found in the patients (table II). In fact, the mean hemoglobin level and transferrin saturation value were 6.4 g/dl and 7.9% respectively.

The serum zinc levels were low in 8 of the 9 patients tested for zinc.

Results of the oral iron tolerance tests are shown for 12 patients and 5 control subjects in figure 4. Patients with iron deficiency anemia associat

Fig 4 Plasma iron tolerance tests. The dotted lines show iron absorption of control subject with iron deficiency anemia, the full lines illustrate the plasma iron tolerance curves of the 12 cases under study.

Fig 5 Plasma zinc tolerance tests. The dotted lines represents zinc concentration in serum of tolerance tests in control subjects while the full lines show plasma zinc tolerance tests in the patients.

in this study contrary to the control subjects who were iron deficient without geophagia, and who had increased iron absorption as was expected from iron deficient individuals. In addition, flat zinc absorption curves were also obtained in the patients with geophagia.

Main differences between these two groups could be summarized as follows. Firstly a long-standing habit of clay eating was present in the 1st group, whereas the 2nd group had simple iron deficiency on a nutritional basis. Secondly the majority of the cases in the patients group who showed iron malabsorption, had zinc deficiency in addition to iron deficiency whereas in the control group iron deficiency was present alone. It has been shown previously that iron deficiency may cause changes in the gastrointestinal mucosa resulting in impaired function. If iron deficiency is taken as a sole factor to be responsible for gastrointestinal disturbances, the difference between the two groups cannot be explained easily. We incline to think that geophagia might be the contributory factor in impaired absorption of iron and zinc. The results of the present study also confirm the findings of Gross *et al* [6] who recently demonstrated an inability to absorb oral iron in 6 patients.

All patients were treated with intramuscular iron and their anemia was corrected.

In view of these findings, it seems reasonable to speculate that the cases with the syndrome characterized by geophagia, iron deficiency zinc deficiency growth retardation, hypogonadism and hepatosplenomegaly observed in Turkey and Iran might have an additional feature of iron and zinc malabsorption.

References

- 1 BERKEL, I, SAY B and KIRAN, O. Intestinal mucosa in children with geophagia and iron-deficiency anemia. *Scand J Haematol* 7: 18-25 (1970).
- 2 BOTHWELL, T. H. and MALLETT. The determination of iron in plasma or serum. *Biochem J* 39: 599 (1955).
- 3 BOTHWELL, T. H. and FRECH, C. A. Iron metabolism, p. 51 (Little, Brown, Boston 1962).
- 4 ÇAYDAR, A. O. and ARCAŞOY, A. Hematologic and biochemical studies of Turkish children with pica. *Clin Pediatr* 11: 215 (1972).
- 5 GOSH, S., DADA, S., KASTHURI, D., MITRA, R. C., and CRUTTAM, H. K. Gastrointestinal function in iron deficiency states in children. *Am J Dis Child* 123: 15 (1977).
- 6 GROSS, S. J., STUART, M. J., SWENDER, E. T. and OCKI, F. A. Malabsorption of iron in children with iron deficiency. *J Pediatr* 5: 795 (1976).

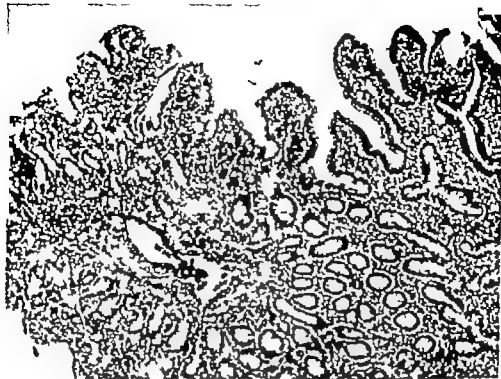


Fig 6. Histopathological findings of duodenal biopsy of case 11 A. h., note the thickening and shortening in villi with increased infiltration in the lamina propria.

ed with geophagia, demonstrated very flat curves against elevated iron absorption curves in control subjects who had simple iron deficiency

Plasma zinc tolerance tests which were also performed in patients and control subjects have been shown in figure 5. The patients had zinc deficiency in addition to iron deficiency whereas control cases were normal. It is clearly visible that the patients had 'flat' zinc absorption curves similar to those seen in iron absorption.

Histopathological analysis of biopsy material revealed significantly shortened, blunted, occasionally fused villi in addition to cellular infiltration in the lamina propria (fig. 6).

Discussion

12 patients with prolonged geophagia and iron deficiency anemia demonstrated an impaired iron absorption through oral iron absorption tests

Platelet Counts in Children with Iron Deficiency Anemia

G. HİÇŞÖNMEZ, K. SÖZER, G. SÖLOĞLU and S. DÖNMEZ

Department of Pediatrics, Hacettepe University Children's Medical Center Ankara

Key Words. Platelet count Iron deficiency anemia Children

Abstract. Platelet count was evaluated in 30 children with iron deficiency anemia. It was found elevated when compared with 40 normal controls. No significant difference was found between the platelet counts in patients with hemoglobin levels higher or lower than 7 g/dl. Although no relation was observed between platelet count and transferrin saturation, it was correlated with serum iron values. After oral and/or parenteral iron therapy platelet count decreased insignificantly while reticulocytes were found to be increased.

Increased and decreased numbers of platelets have both been documented in children and adults with iron deficiency anemia of different causes [3, 6, 9, 10]. Because of controversial results, we studied the platelet counts of 30 children with nutritional iron deficiency anemia whose folic acid levels were normal. Platelet counts of these patients were also reevaluated following iron treatment.

Material and Methods

30 children with iron deficiency anemia, with the age range of 6 months to 3 years (11 27 and 6, 8, and 13 years in 3), were the subjects of this study.

The diagnoses of iron deficiency anemia was established in all cases by the decreased transferrin saturation values ($< 15\%$) and low hemoglobin (Hb) concentration (< 10 g/dl) in the presence of hypochromia, reticulocytopenia ($< 1\%$) and poor nutritional history. No evidence of infection was found in any of these patients and the physical findings were not contributory. Also, there was no history of blood loss.

- 7 GUHA, D. K., WALLA, B. N., TANDON, B. N. *et al.* Small bowel changes in iron deficiency anemia of children. *Archs Dis. Childn.* 43 239-244 (1968)
- 8 HALSEAD, A. A., PRASAD, A. S., and NADIMI, M. Gastrointestinal function in iron deficiency anemia. *Archs Intern. Med.* 116 253-256 (1965).
- 9 KIMBER, C. and WENTROB, L. R. Malabsorption of iron secondary to iron deficiency. *New Engl. J. Med.* 276 453 (1968).
- 10 MENNICH, V., OKÇUOĞLU, A. and ARCAŞOY, A. Pica in Turkey: the effect of clay upon iron absorption. *Am. J. clin. Nutr.* 21 78 (1968)
- 11 NADMAN, J. L., OSKI, F. A., DIAMOND, L. K., WAWTER, G. F. and SCHWACIDMAN, H. The gastrointestinal effects of iron deficiency anemia. *Pediatrics*, Springfield 33 83 (1964)
- 12 NATHAN, D. G. and OSKI, F. A. Hematology of infancy and childhood, p. 102 (Saunders, Philadelphia 1974)
- 13 Perkin-Elmer. Clinical methods for atomic absorption spectroscopy: determination of copper in serum, determination of zinc in serum (Perkin Elmer Norwalk 1971).

Platelet Counts in Children with Iron Deficiency Anemia

G. HİÇSÖNMEZ, K. SÖZER, G. SÜLOĞLU and S. DÖNMEZ

Department of Pediatrics, Hacettepe University Children Medical Center Ankara

Key Words. Platelet count Iron deficiency anemia Children

Abstract. Platelet count was evaluated in 30 children with iron deficiency anemia. It was found elevated when compared with 40 normal controls. No significant difference was found between the platelet counts in patients with hemoglobin levels higher or lower than 7 g/dl. Although no relation was observed between platelet count and transferrin saturation, it was correlated with serum iron values. After oral and/or parenteral iron therapy platelet count decreased insignificantly while reticulocytes were found to be increased.

Increased and decreased numbers of platelets have both been documented in children and adults with iron deficiency anemia of different causes [3 & 9-10]. Because of controversial results, we studied the platelet counts of 30 children with nutritional iron deficiency anemia whose folic acid levels were normal. Platelet counts of these patients were also reevaluated following iron treatment.

Material and Methods

30 children with iron deficiency anemia, with the age range of 6 months to 3 years in 27 and 6, 8, and 13 years in 3, were the subjects of this study.

The diagnosis of iron deficiency anemia was established in all cases by the decreased transferrin saturation values ($< 15\%$) and low hemoglobin (Hb) concentration (< 10 g/dl) in the presence of hypochromia, reticulocytopenia ($< 1\%$) and poor nutritional history. No evidence of infection was found in any of these patients and the physical findings were not contributory. Also, there was no history of blood loss.

or drug administration which might have affected the platelet count. Stool guaiac tests were found negative on at least two occasions. 40 healthy children with normal hemoglobins, ranging in age from 6 months to 3 years were studied as controls.

Effect of iron therapy on platelet counts was evaluated in 12 iron deficient children during oral and parenteral (i.m.) iron therapy in two divided groups. Oral iron, as ferrous sulfate, 6 mg/kg/day of elemental iron was given to 6 patients. Follow-up Hb and Hct determinations, reticulocyte and platelet counts were obtained every 3 days for a 30-day period. Parenteral iron in doses calculated to correct anemia and to replenish iron stores were given intramuscularly. Follow up determinations were carried out every other day for 2 weeks.

Routine hematologic methods were used for hemoglobin and reticulocyte counts. Platelet count was evaluated on the phase microscope by the methods of BRECHER and CRONKITE [1]. Serum iron (SI), serum iron-binding capacity and folic acid were determined by the methods of SHAWMONS [11] (modified), PETERS *et al.* [1] and HERBERT [7], respectively.

Results

The mean hemoglobin value of 30 iron-deficient children was 7.25 ± 0.26 g/dl (range 5–9.75 g/dl) serum iron was 30.55 ± 2.64 μ g/dl (range 10–62 μ g/dl) transferrin saturation was $7.51 \pm 1.43\%$ (range 2.35–14%) and folic acid was 18.07 ± 1.88 ng/ml (range 7.15–45 ng/ml). The platelet counts of the patients and controls are shown in table I. Significantly elevated platelet counts were found in the study group ($p < 0.05$). The distribution of platelet counts and hemoglobin values in patients and controls can be seen in figure 1. No correlation was observed between platelet counts and hemoglobin levels. However the platelet counts of patients whose serum iron was below 20 μ g/dl were found to be significantly higher than the platelet counts of patients with serum iron above 20 μ g/dl ($p < 0.05$ table II).

During the course of oral and parenteral iron therapy in 12 children the platelet count was found to be decreased insignificantly while the reticulocytes were found to be increased. Platelet count returned to the pre treatment level on the 15th day and did not change much during the follow-up period.

Discussion

An increase in the platelet count after acute and chronic blood loss has been well documented [5]. SCHLOESSER *et al.* [10] reported increased number of platelets in the majority of their iron-deficient children with

Table I Platelet counts in 30 children with iron deficiency anemia and controls

	Platelet counts/pl patients	controls
Mean \pm SD	375,000 \pm 15,750*	272,595 \pm 25,866
Range	236,000 - 644,000	160,000 - 418,000

*Significantly different from controls ($p < 0.05$)

Table II Correlation of platelet counts according to the value of hemoglobin, serum iron, and transferrin saturation in iron deficiency anemia

	Hemoglobin, g/dl		Serum iron, μ g/dl		Transferrin saturation, %	
	≥ 7	< 7	≥ 20	< 20	≥ 7	< 7
Number of patients	16	14	11	7	15	11
Platelet counts/pl						
Mean	385,000	364,285	362,000	436,571	353,000	302,000
\pm SD	26,363	16,362	12,066	25,530	21,734	35,142
p		0.05		< 0.05		> 0.05



Fig 1 The distribution of platelets and hemoglobin values in children with iron deficiency anemia, and controls (mean and SD of platelet counts is indicated). A Patients. B Controls.

or drug administration which might have affected the platelet count. Stool guaiac tests were found negative on at least two occasions. 40 healthy children with normal hemoglobins, ranging in age from 6 months to 3 years were studied as controls.

Effect of iron therapy on platelet counts was evaluated in 12 iron deficient children during oral and parenteral (I.M.) iron therapy in two divided groups. Oral iron, as ferrous sulfate, 6 mg/kg/day of elemental iron, was given to 6 patients. Follow-up Hb and Hct determinations, reticulocyte and platelet counts were obtained every 3 days for a 30-day period. Parenteral iron in doses calculated to correct anemia and to replenish iron stores were given intramuscularly. Follow up determinations were carried out every other day for 2 weeks.

Routine hematologic methods were used for hemoglobin and reticulocyte counts. Platelet count was evaluated on the phase microscope by the methods of BRECHER and CROCKETT [1]. Serum iron (SI), serum iron-binding capacity and folic acid were determined by the methods of SHUMWAY [11] (modified), PETERS *et al* [12] and HERBERT [7], respectively.

Results

The mean hemoglobin value of 30 iron-deficient children was 7.25 ± 0.26 g/dl (range 5–9.75 g/dl) serum iron was 30.55 ± 2.64 μ g/dl (range 10–62 μ g/dl) transferrin saturation was $7.51 \pm 1.43\%$ (range 2.35–14%), and folic acid was 18.07 ± 1.88 ng/ml (range 7.15–45 ng/ml). The platelet counts of the patients and controls are shown in table I. Significantly elevated platelet counts were found in the study group ($p < 0.05$). The distribution of platelet counts and hemoglobin values in patients and controls can be seen in figure 1. No correlation was observed between platelet counts and hemoglobin levels. However the platelet counts of patients whose serum iron was below 20 μ g/dl were found to be significantly higher than the platelet counts of patients with serum iron above 20 μ g/dl ($p < 0.05$ table II).

During the course of oral and parenteral iron therapy in 12 children the platelet count was found to be decreased insignificantly while the reticulocytes were found to be increased. Platelet count returned to the pre-treatment level on the 15th day and did not change much during the follow up period.

Discussion

An increase in the platelet count after acute and chronic blood loss has been well documented [5]. SCHLOESSER *et al* [10] reported increased number of platelets in the majority of their iron-deficient children with

plate conditions. Recently, it has been shown that partially purified preparations of erythropoietin and thrombopoietin may be capable of stimulating thrombopoiesis if used in sufficient quantities [4].

Although there are some studies concerning the mechanism of thrombocytosis it still remains unsolved in iron deficiency anemia.

Acknowledgements

We are indebted to Prof. S. Özcan for his help in reviewing the manuscript and to Mrs. E. BALKAS for editorial assistance.

References

1. BAUCHER, H. and CROWNETZ, E. F. Morphology and enumeration of blood platelets. *J. appl. Physiol.* 3: 365-377 (1950).
2. CAROL, S. and SNEAD, J. V. Platelet production in experimental iron deficiency anemia. *Blood* 42: 219-228 (1973).
3. DEMICEL, K. and ARIOY, M. On the platelet levels in chronic iron deficiency anemia. *Acta haemat.* 41: 135-143 (1969).
4. EVITT, B. L., SPIVAK, J. L. and LEVY, J. Relationships between thrombopoiesis and erythropoiesis; with studies of the effects of preparations of thrombopoietin and erythropoietin. *Blood* 43: 547-558 (1976).
5. GARG, S. K., WEINER, M. and KARPSTEIN, S. Thrombocyte and Megathrombocyte kinetics during thrombocytosis induced by acute and chronic blood loss and by iron deficiency diet. *Haemostas. J.* 121-135 (1973).
6. GROSS, S., KEEFER, V. and NEWMAN, A. J. The platelets in iron deficiency anemia. I. The response to oral and parenteral iron. *Pediatrics*, Springfield 34: 315-323 (1964).
7. HERBERT, V. The assay and nature of folic acid activity in human serum. *J. clin. Invest.* 40: 81-91 (1961).
8. JACKSON, C. W., SNEAD, J. V. and CAROL, E. The relationship of anemia and thrombocytosis. *J. Lab. clin. Med.* 64: 357-368 (1974).
9. LOPAS, H. and RABNER, S. F. Thrombocytopenia associated with iron deficiency anemia. A report of five cases. *Chil. Pediatr.* 3: 609-616 (1966).
10. SCHLOSSER, L. L., KIPP, M. A. and WENZEL, F. J. Thrombocytosis in iron deficiency anemia. *J. Lab. clin. Med.* 66: 107-114 (1965).
11. SNYDER, J. S. Determinations of serum iron. *Med. Publ. Health Lab. Meth.*, 6th ed. p. 416 (Len & Febiger Philadelphia 1955).
12. PETERS, T., GIOVINELLO, T. J., APT, L. and ROSS, J. R. A new method for the determination of serum iron binding capacity. *J. Lab. clin. Med.* 40: 274-279 (1956).

G. HANCIÖRÖZ, Department of Pediatrics, Hacettepe University Children's Medical Center Ankara (Turkey)

the evidence of blood loss. Thrombocytopenia as well as thrombocytosis have been reported in children with iron deficiency anemia [6-9]. Low platelet counts were observed in adults with iron deficiency anemia who had hypersplenism [3]. However, in the same study no difference was found between the controls and the patients who had iron deficiency anemia due to any other cause except hypersplenism. The conflicting results found in the literature might be caused by the heterogeneity of the patients studied.

In our study of children with nutritional iron deficiency anemia who had no bleeding, folic acid deficiency, infection, hypersplenism or any other factors which might have affected the platelet counts, platelet counts were found significantly higher than in controls. Although Gross *et al* [6] described thrombocytosis ($420\,000/\mu\text{l}$) in children with hemoglobin concentrations above 6 g/dl, thrombocytopenia was observed in children with mean hemoglobin values of 4 g/dl. In our patients the mean hemoglobin and platelet values were 7.25 g/dl and $375\,000 \pm 15\,750/\mu\text{l}$ respectively and no thrombocytopenia was observed. The difference in platelet counts between Gross *et al*'s and our findings might be explained by the severity of the anemia and presence of other factors in their patients. An inverse relationship between platelet counts and hematocrit in an iron-deficient animal was shown by Choi and Simone [2]. Jackson *et al* [8] indicated the necessity of critically lowering hematocrit values for the stimulation of megakaryocytopoiesis. In this study no significant relationship was shown between platelet counts and hemoglobin levels below or above 7 g/dl. Although no significant relation was shown between platelet counts and transferrin saturation, it was correlated with serum iron values (table II). This result might be explained by Schloesser *et al*'s [10] statements that the thrombocytosis in iron deficiency anemia could be related to the low level of serum or tissue iron. After iron therapy decline in platelets has been shown in patients and experimental studies previously [2, 6-10]. In our patients insignificant reduction in platelet counts was observed with the replacement therapy.

The relationship between the abnormal platelet count and iron deficiency anemia was studied by Choi and Simone [2]. They suggested that shortened maturation time and/or increased polyploidy of megakaryocytes might be the cause of thrombocytosis. They found normal platelet survival. Previously prolongation of platelet survival was thought to be the cause of thrombocytosis. Jackson *et al* [8] indicated that erythropoietin may cause increased platelet production in anemia under appro-

prime conditions. Recently it has been suggested that the use of preparations of erythropoietin and thrombopoietin may be of value in stimulating thrombopoiesis if used in sufficient quantities [4].

Although there are some studies concerning the mechanism of thrombocytosis it still remains unsolved in iron deficiency anemia.

Acknowledgements

We are indebted to Prof S. ÖZSOYLU for his help in reviewing the manuscript and to Mrs. E. BALKAS for editorial assistance.

References

1. BRECHER, G. and CRONSTE, E. F. Morphology and enumeration of blood platelets. *J Appl. Physiol.* 3: 363-377 (1950).
2. CHOI, S. and SHAGAR, J. V. Platelet production in experimental iron deficiency anemia. *Blood* 42: 219-228 (1973).
3. DE-COL, K. and ARNOY, M. On the platelet levels in chronic iron deficiency anemia. *Acta haemat.* 41: 135-143 (1969).
4. EVATT, B. L., SPRYAK, J. L., and LEVY, J. Relationships between thrombopoiesis and erythropoiesis with studies of the effects of preparations of thrombopoietin and erythropoietin. *Blood* 48: 547-558 (1976).
5. GARG, S. K., WEINER, M. and KAPTEIN, S. Thrombocyt and Megathrombocyte kinetics during thrombocytosis induced by acute and chronic blood loss and by iron deficiency diet. *Haemostas* 1: 121-135 (1973).
6. GROSS, S., KEEFER, V. and NEWELL, A. J. The platelets in iron deficiency anemia. I. The response to oral and parenteral iron. *Pediatrics*, Springfield 34: 315-323 (1964).
7. HERBERT, V. The assay and nature of folic acid activity in human serum. *J. clin. Invest.* 40: 81-91 (1961).
8. JACKSON, C. W., SEWOME, J. V. and CAROL, E. The relationship of anemia and thrombocytosis. *J. Lab. clin. Med.* 64: 357-368 (1974).
9. LOPAS, H. and RABNER, S. P. Thrombocytopenia associated with iron deficiency anemia. A report of five cases. *Clin. Pediatr.* 5: 609-616 (1966).
10. SCHLOESSER, L. L., KIPP, M. A., and WENZEL, H. J. Thrombocytosis in iron deficiency anemia. *J. Lab. clin. Med.* 66: 107-114 (1965).
11. SEWOME, J. S. Determinations of serum iron. *Med. Publ. Health. Lab. Meth.* 6th ed. p. 416 (Lee & Febiger Philadelphia 1955).
12. PETERS, T., GIOVANELLO, T. J., APT, L., and ROSS, J. A new method for the determination of serum iron binding capacity. *J. Lab. clin. Med.* 45: 774-779 (1956).

Congenital Hypoplastic Anaemia Refractory to Corticosteroids but Responding to Cyclophosphamide and Antilymphocytic Globulin

Report of a Case Having Responded with a Transitory Wave of Dyserythropoiesis

A. M. MARMONT

Division of Haematology and Clinical Immunology Ospedale Generale Regionale, Genova

Key Words Anaemia · Immunosuppression · Antilymphocytic globulin · Cyclophosphamide

Abstract A 7 year-old male child with congenital hypoplastic anaemia (Diamond Blackfan), having originally responded to corticosteroids, but having subsequently become refractory and erythroblastopenic since 4 years, was treated with a combination of cyclophosphamide (CY 2.1 g) and antilymphocytic globulin (ALG 8 g), both by the intravenous route. Erythroblastic repopulation of the bone marrow albeit dyserythropoietic in character reticulocytosis and erythrocytic increment took place but only for a short period. The significance of these findings is discussed in the light of recent progress in the understanding of the disease and of its treatment.

First accurately described by DIAMOND and BLACKFAN [6] in 1938, congenital hypoplastic anaemia (CHA) is an infrequent disorder of the blood occurring in young children in which severe aplasia of the erythron is associated with an extreme and specific erythropoietic failure. Some 200 cases have been reported until 1976 according to HARDISTY [13] with whom I agree in considering that the most appropriate name for the disease would be congenital pure red cell aplasia thus associating it with the equally selective pure red cell aplasia (PRCA) of the adult and distinguishing it from nonselective, panhaemocytopenic aplasias.

CHA has been considered for decades a congenital defect with a fail

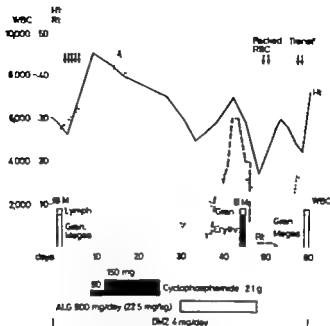


Fig 1 Haematologic course of patient during and after immunosuppressive treatment. Note the marked fall of WBCs after CY, the subsequent reticulocyte peak and erythrocyt increment during the administration of ALG, and the fall of both these parameters soon after. The patient was then given packed red cell transfusions.

ure of the organ itself perhaps with an autosomal recessive inheritance [5] but the whole pathogenetic outlook has altered considerably as a consequence of recent immunological methods. Although the presence of a humoral, antibody-like inhibitor of erythropoiesis, as reported in 9 cases by ORTEGA *et al* [27] was not confirmed subsequently [10] a subpopulation of peripheral blood lymphocytes, that is erythroid-suppressor lymphocytes, has been shown to be a primary agent in the production of erythroblastic aplasia by HOFFMANN *et al* [14].

Corticosteroids have been the mainstay of treatment for the great majority of these patients, however many children fail to respond, either primarily or subsequently and become candidates for regular blood transfusions, which carry with them the well known hazard of iron overload. A

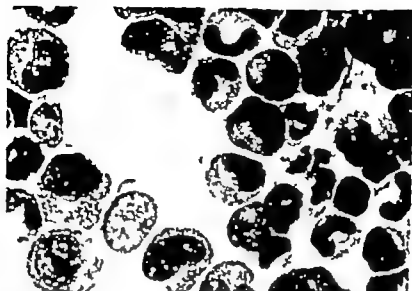


Fig 2 Sternal bone marrow aspirate before treatment. Granulocytic hyperplasia is prominent, but no red cell precursors are visible. A markedly immature cell is seen at right centre.

bone marrow transplantation from a histocompatible sibling was performed in a patient having received 238 transfusions [3].

However, also before the recent pathogenetic insights briefly discussed above, at least 2 cases of steroid refractory CHA had been treated successfully with the antimetabolite immunosuppressant 6-mercaptopurine (6-MP) at the ages of 15 months [30] and 5 years [33] respectively. The purpose of this paper is to report an additional case having responded to potent combination immunosuppressive therapy when in an advanced corticosteroid refractory stage; however, the erythropoietic response was transitory and markedly dyserythropoietic in character.

Case Report

At birth (September 1967) the patient was a waxy pale infant with extremely severe anaemia (0.9×10^6 /l RBCs). He was immediately transfused and had a fair recovery. At the age of 4 months, sternal aspiration showed complete absence of erythroblasts with an otherwise normal picture. A diagnosis of CHA (Diamond Blackfan) was made, and the infant was put on a regular corticosteroid treatment; a satisfactory remission took place, and at 1 year of age his RBCs were 3×10^6 /l and a discrete erythroblastosis could be found in the bone marrow.

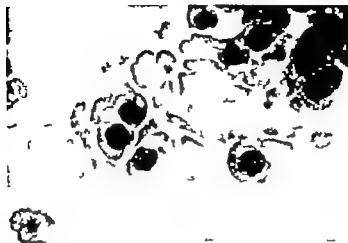


Fig 3 Orthochromatic erythroblasts after treatment. Nuclear contours are blurred, Jolly-like bodies are present in the cytoplasm and intercellular connections are also visible



Fig 4 Composite photomicrograph showing nuclear fragmentation of orthochromatic erythroblasts

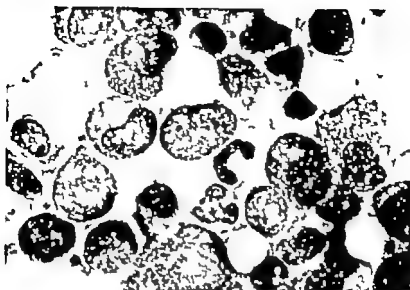


Fig 5 Bone marrow relapse with predominantly immature granulocytic hyperplasia. Erythroblasts are once again completely absent.

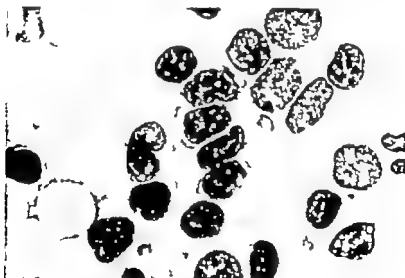


Fig 6. Focal lymphocytosis in the bone marrow aspirate at time of relapse

The patient responded to corticosteroids from 1968 to 1971 however from October 1972 to January 1975 no erythroblasts could be found in the marrow and 2 blood transfusions per month were found to be necessary to maintain a level of 2×10^4 RBCs. On February 24, 1975 he was referred to our Division in view of



Fig 7 Focal bone marrow lymphocytosis with bizarre monocytoid aspects (activated lymphocytes?).

the results obtained with the immunosuppressive treatment of adult PRCA [20, 21, 23-25].

Informed consent to this type of treatment was given by both parents and the attending family paediatrician, who in fact had suggested it.

Physical examination showed somewhat scarcely developed, pale male child, but no skeletal or somatic abnormalities were noted. The heart and spleen were enlarged, the liver edge was distinctly palpable, but not excessively firm.

Blood examinations showed $3 \times 10^{12}/l$ RBCs, $5.2 \times 10^9/l$ WBCs with normal differential and $250 \times 10^9/l$ platelets. Reticulocytes were quite absent. Sternal aspiration showed high cellularity composed of granulocyte precursors, megacaryocytes and small lymphocytes ($< 10\%$). Erythroblasts were completely absent. All other tests and investigations were negative or non-contributory.

After 2 packed erythrocyt transfusions, administered because of progressive anaemia during the investigational period, the patient, who had always been kept on 4 mg of dexamethasone/day was treated with cyclophosphamide (CY), 2.05 g administered intravenously over a period of 11 days, starting with 50 mg/day for 2 days and following with 150 mg/day for another 10. This treatment was followed by fall of WBC to $0.7 \times 10^9/l$. After 10 day interval, during which reticulocytes were always absent, ALG was administered, also intravenously at dosage of 400 mg/day (22.2 mg/kg) for 16 days, totalling 8 g. On day 6 from the start of ALG, the first reticulocytes appeared, attaining peak of 3.2% on day 12. A increment

Behringwerke ALG was employed throughout this treatment. It is of equine origin and contains 50 mg/ml of immunoglobulins, which are composed of IgG and IgM with at least 90% of the total protein being 7 S immunoglobulins.

from 0.28 to 0.38 in the haematocrit (PCV) followed. Sternal aspiration on day 14 showed an intense erythroblastosis. Binucleated erythroblasts, cytoplasmatic connections, interchromatin bridges, blurred and irregular nuclear outlines, nuclear budding and fragmentation were prominent. Also intercellular clear junctions of the so-called syncytial type [7] were sometimes observed.

7 days after the peak blood reticulocytes had again disappeared the haematocrit fell abruptly to 0.23 and transfusions were again necessary. Sternal aspiration, performed 15 days after completion of ALG treatment, showed a total disappearance of erythroblasts, marked granulocytoblastic hyperplasia and lymphocytic infiltration, often with some aspects of activation.

In view of the transitory and insufficient character of this remission further immunosuppressive treatment was not programmed any more. The patient was discharged, he is living and in good clinical condition at the time of this writing, but has to be transfused regularly.

Discussion

Even with the exclusion of enzyme-deficient [11] and relapsing [12] erythroblastopenias, CHA has been considered a heterogeneous condition with possibly genetic and acquired subgroups [13]. While the first would appear to be best associated with developmental anomalies, the evidence for an autoimmune pathogenesis of the second is quite impressive. Suppressor lymphocytes were first postulated as acting on B subpopulations of lymphocytes [1] that is, as a part of the so-called immunological network [17] but it is now recognized that they may act upon a variety of cellular targets by means of soluble mediator molecules [32]. Besides CHA suppressor lymphocytes have been implicated in the pathogenesis of aplastic anaemia [2-15] and, even in adult PRCA in which an impressive evidence is in favor of pathogenetic IgG autoantibodies [16-25, 28] the possible significance of bone marrow lymphocytosis has been commented upon repeatedly [20-21, 25].

The therapeutic effect of corticosteroids in CHA has been explained by the correction of some enzymatic abnormality [5] of the erythroid precursor cells, but an immunosuppressive effect can be equally envisaged. Moreover such an effect is undisputable with specific cytotoxic agents such as 6-MP, CY and/or ALG. Although antilymphocytic globulin raised against predominantly T cell subpopulations (ATG) is considered as anti-T selective, ordinary ALG is also capable of acting on T lymphocyte targets [23-24]. Clear-cut therapeutic effects with ALG have been obtained in PRCA of the adult [20-21, 25] and in severe idiopathic aplastic anaemia [31].

The markedly dyserythropoietic morphological features of the transitory erythroid response in this patient are also of some interest. Dyserythropoiesis of greater or lesser severity occurs in a wide range of conditions [18, 19]. It is quite frequent in aplastic anaemia [8, 9] but has not been described neither in PRCA nor in CHA, although a condition 'intermediate' between the latter and the congenital dyserythropoietic anaemias has also been described recently [26]. Dyserythropoiesis has been reported in fetal hepatic erythropoiesis [29] and in early post-transplantation haematopoiesis [22] and has been considered, together with the production of fetal haemoglobin [3] as a partial and accelerated recapitulation of ontogeny. In this case dyserythropoiesis did not develop into orthoerythropoiesis, as in the successful bone marrow transplant [22] but was followed by a complete failure of red cell production, thus reflecting the advanced and practically irreversible evolution of the disease. It is perhaps in such a stage that bone marrow transplantation from a compatible sibling should be contemplated [3] since any autoimmune mechanism is abolished, perhaps irreversibly by massive cyclophosphamide conditioning.

References

- 1 ALLISON, A. C. Mechanism of tolerance and autoimmunity. *Ann. rheum. Dis.* 32: 283-293 (1973).
- 2 ASCENLAND, J., PARWA, R., KAGAN, W., HAUSER, J., MOORE, M., and GOOD, R. A. Aplastic anaemia. Evidence for an immunological mechanism. *Lancet* i: 669-671 (1976).
- 3 ADGUST, C. S., KING, E., GITHENS, J. H., MCINTOSH, K., HUMBERT, J. R., GREENBERG, A. and JOHNSON, F. B. Establishment of erythropoiesis following bone marrow transplantation in a patient with congenital hypoplastic anaemia (Diamond-Blackfan syndrome). *Blood* 1976: 491-498.
- 4 BARAN, D. T., GROSS, P. F. and KLEMPERER, M. R. Recovery from aplastic anaemia after treatment with cyclophosphamide. *New Engl. J. Med.* 295: 1522-1523 (1976).
- 5 DIAMOND, L. K. Congenital hypoplastic anaemia; in METCALF, CORDONELLI and PERCELE. *Leukemia and aplastic anemia*, pp. 45-50 (Il Pensiero Scientifico, Roma 1976).
- 6 DIAMOND, L. K. and BLACKFAN, K. H. Hypoplastic anaemia. *Am. J. Dis. Child* 36: 464-466 (1938).
- 7 FLANDRIN, O., DANIEL, M. T., BRETON-GORIUS, J., BROUET J.-C. et BERNARD, J. Un érythroblastique anormal dû au développement de jonctions intercellulaires (syndrome érythroblastique). Un nouveau mécanisme d'origine. Problèmes posés par le diagnostic. *Nouv. Revue fr. Hémat.* 14: 161-180 (1974).

- 8 FRISCH, B. and LEWIS, S. M., The bone marrow in aplastic anaemia. Diagnostic and prognostic features. *J. clin. Path.* 27 231-241 (1974)
- 9 FRISCH, B. LEWIS, S. M. and SHERMAN B. The ultrastructure of dyserythropoiesis in aplastic anaemia. *Br J Haemat.* 29 545-549 (1975).
- 10 GELLER, G. KRIVIT W. ZALUSKY R., and ZANJANI, E. D. Lack of erythropoietic inhibitory effect of serum from patients with congenital pure red cell aplasia. *J. Pediat.* 2 198-201 (1975)
- 11 GILLET E. ANDMANN, A. J. WARA, D. SANDMAN R., and DIAMOND, L. K., Nucleoside phosphorylase deficiency in a child with severely defective T-cell immunity and normal B-cell immunity *Lancet* i, 1010-1013 (1975).
- 12 GIROT R. et GRACILLI, C., Erythroblastopénie à rechutes. A propos d'un cas suivi pendant 11 ans. *Nouv. Revue fr. Hémat.* 18 555-562 (1977).
- 13 HARDISTY R. M., Diamond-Blackfan anaemia in *Congenital disorders of erythropoiesis*, pp. 89-96 (Elsevier/Excerpta Medica/North-Holland, Amsterdam 1976)
- 14 HOFFMAN R. ZANJANI, E. D. ZALUSKY R. LUTTON J. D. and WASSERMAN L. R. Diamond Blackfan syndrome. Lymphocyte-mediated suppression of erythropoiesis. *Science* 193 899-900 (1976)
- 15 HOFFMAN, R., ZANJANI, E. D., LUTTON J. D. ZALUSKY R., and WASSERMAN, L. R. Suppression of erythroid-colony formation by lymphocytes from patients with aplastic anemia. *New Engl. J. Med.* 296, 10-13 (1977).
- 16 KRANTZ, S. B. Annotation. Pure red cell aplasia. *Br J Haemat.* 25 1-6 (1973).
- 17 JERNE, N. K., The immune system. A network of lymphocyte interactions in MELCHERS and RAJEWSKY *The immune system*, pp.259-266 (Springer Berlin 1976)
- 18 LEWIS, S. M. and VERWILGEN, R. L., Dyserythropoiesis and dyserythropoietic anemias in BROWN *Progress in hematology* vol. 8 pp 99-129 (Grune & Stratton, New York 1973)
- 19 LEWIS, S. M. and VERWILGEN R. L., Dyserythropoiesis: definition, diagnosis and assessment in LEWIS and VERWILGEN *Dyserythropoiesis*, pp. 3-20 (Academic Press, London 1977)
- 20 MARMONT A., Pure erythroblastopenia or red cell aplasia as an autoimmune disease responding to immunosuppressive therapy in LIBANSKY DOENNER *Present problems in hematology* p. 225 (Excerpta Medica, Amsterdam 1974)
- 21 MARMONT A., Il trattamento immunosoppressivo della eritroblastopenia pura per mezzo di ciclofosfamido e di globuline antilinfocitarie. *Minerva Med.* 66. 2490-2508 (1975).
- 22 MARMONT A., DAMASIO, E. BACIGALUPO, A., GIORDANO, D., ROSSI, E. REALI, G. GAY A., DAGNA BRACARELLI, F. BREMA, F. CARELLA, M., and SANTINI, G. A to O bone marrow transplantation in severe aplastic anaemia. Dynamics of blood group conversion and demonstration of early dyserythropoiesis in the engrafted marrow *Br J Haemat.* 36. 511-518 (1977).
- 23 MARMONT A., DAMASIO, E., GIORDANO, D. CARELLA, M. SANTINI, G. and BACIGALUPO, A., The treatment of autoimmune blood diseases with antilymphocytic globulin. *Post-grad. med. J.* 52 suppl. 5, pp 139-146 (1976).

- 24 MARMONT A., DAMASO, E., GIORDANO, D., SANTINI, G., CARELLA, M., BACCALUPO, A. ROSSI, E. Il trattamento delle autoimmunopatie con \equiv immunoglobuline antileucocitarie. Atti XXV Congr. Naz. Soc. It. Ematol. 2. 707-816 (1975).
- 25 MARMONT A., PERCHLE, C., SANGUINETTI, M. and CONDORELLI, M. Pure red cell aplasia (PRCA). Response of three patients to cyclophosphamide and/or antilymphocytic globulin (ALG) and demonstration of two types of serum inhibitors to erythropoiesis. *Blood* 45. 247-261 (1975).
- 26 NERI, A. Congenital hypoplastic anaemia with unusual dyserythropoietic features. *Acta haemat.* 53. 278-287 (1977).
- 27 ORTEGA, J. A., SMORE, M. A., DUKES, P. P. and HANSEN, D. Congenital hypoplastic anemia. Inhibition of erythropoiesis by sera from patients with congenital hypoplastic anemia. *Blood* 45. 83-89 (1975).
- 28 PERCHLE, C. Regulation of erythropoiesis and its defects. *Br J Haemat.* 31 suppl. pp. 69-81 (1975).
- 29 QUATTER, N., DOD, E., and ROSA, L. DE: Dyserythropoietic features of human foetal erythropoiesis and their significance; in METCALF CONDORELLI and PERCHLE Leukemia and aplastic anemia, pp.281-285 (Il Pensiero Scientifico, Roma 1976).
- 30 SCHÖLER, J. BOCHER, J. und KLEFMAN, E. Genesung einer chronischen Erythrozytenaplasie während einer Behandlung mit 6-Merkeptopurin. *Kinderärztl. Prax.* 33. 145-147 (1970).
- 31 SMICK, B., BUCKNER, C. D. CONY, P. and JEANGUY M. Rationale for the use of ALG as sole immunosuppressant \equiv allogeneic bone marrow transplantation for aplastic anemia. *Transplants Proc.* 8. 617-622 (1976).
- 32 WAKSMAN, B. H. and NAMBA, Y. Commentary On soluble mediators of immunologic regulation. *Cell Immunol.* 21. 161-176 (1976).
- 33 WILANDZ, L., BOGOVIER, J. O. KELLANDER, A., and KELLANDER, J. Pure red cell anemia with pro-erythroblast maturation arrest. *Scand. J Haematol.* 7. 73-76 (1970).

Serum Lipid Pattern in β -Thalassaemia

OMERO GIARDINI, FABRIZIO MURGIA, FRANCESCO MARTINO,
OLGA MANNARINO GIOVANNI CORRADO and GIORGIO MAGGIORI

Istituto di Clinica Pediatrica I Cattedra (Director: E. REZZA)

della Università di Roma, Roma, and

Istituto di Puericoltura (Director: G. MAGGIORI) dell'Università di Sassari Sassari

Key Words: Thalassaemia · Peroxidation · Serum lipids · Polyunsaturated fatty acids · Tocopherols

Abstract Serum lipids, phospholipid fractions and the composition of serum lipid fatty acids were studied in 20 children presenting β -thalassaemia major, 20 heterozygous children and 20 normal controls. Total serum phospholipids, their fractions and cholesterol were significantly lower in patients with thalassaemia major. These changes were referred to hepatic damage and to severe anaemia, respectively. Some serum lipid polyunsaturated fatty acids were significantly decreased in patients with thalassaemia major as compared to heterozygotes and normal controls. Since these alterations are a sign of lipid oxidation, the causes of this phenomenon are discussed.

The cause of diminished erythrocyte survival and subsequent haemolysis in thalassaemia major is not completely known.

According to STOCKS *et al.* [27] increased susceptibility to haemolysis of thalassaemic erythrocytes could be related to lipid peroxidation of the red cell membrane, as evidenced by the increase in production of malonyldialdehyde (an intermediate product of lipid peroxidation) after oxidative stimulation. This phenomenon is revealed in the red blood cell by the decrease in polyunsaturated fatty acids contained mostly in phosphatidylserine and phosphatidylethanolamine [6].

We are grateful to Dr. CARLO CHIARAMONTE for his statistical aid.

According to JACOB and LUX [15] the first sign of oxidative haemolysis is a decrease in erythrocyte phosphatidylethanolamine. Previously [10, 17] we demonstrated a reduced concentration of phosphatidylserine and phosphatidylethanolamine and of some long-chain unsaturated fatty acids in erythrocytes of β -thalassaemic homozygotes, as compared to normal subjects of the same ethnic origin (Northern Sardinia). These results, which suggest a peroxidation of erythrocyte membrane lipids, were subsequently confirmed by other authors [23].

It is still to be clarified whether the oxidative haemolysis, consequent to the peroxidation phenomenon, is due to metabolic alterations in erythrocytes, plasma factors, or both.

If lipid peroxidation in thalassaemia were due totally or in part to extrinsic RBC factors, a serum lipid change in these subjects should be expected. Therefore, we studied serum lipids and their fatty acid composition in homo- and heterozygous β -thalassaemic patients and in normal subjects of the same ethnic origin.

Materials and Methods

Total serum cholesterol, triglycerides, total phospholipids and their fractions as well as serum lipid fatty acid composition were determined in 20 β -thalassaemic homozygotes (9 males, 11 females), in 20 β -thalassaemic heterozygotes (12 males, 8 females) and in 20 normal subjects (11 males, 9 females): all of the above three groups were in the paediatric range (homozygotes and normal subjects 1-4 years, heterozygotes 3-9 years old) and of the same ethnical origin (Northern Sardinia).

Triglycerides were determined by the method of FLETCHE [9], total phospholipids by the method of BAGGESS *et al.* [1] and total cholesterol by the enzymatic method of ROSENBLAU *et al.* [24].

Phospholipid fractions were separated by thin-layer chromatography according to SKRISKE *et al.* [26]. Quantitative determination of the fractions was performed by the method of BAGGESS *et al.* [3], directly on the silica gel scraped from the plate. The detection of phosphates on chromatograms was performed according to ROSENBERG [25].

2,6-Di-*tert*-butyl-*p*-cresol was added to all reagents for extraction and for thin-layer chromatography to avoid autooxidation [7]. Phospholipid fractions were expressed in micrograms of phospholipid-P at 0.1 ml of plasma. Serum fatty acid composition was studied by gas chromatography employing Carlo Erba gas chromatograph 'Fractovap model IV' with flame ionization detector and polyester column (EGGS 10⁶ in Gaschrom 100-120 mesh). Serum lipid extraction was performed by the method of BAGGESS [4]. Fatty acid methylation was carried out according to JAMES *et al.* [16]. The column temperature was monitored at 120-10 °C with temperature increase rate of 3 °C/min. Pure nitrogen was

employed as the carrier gas. Fatty acids were identified by internal standard, peaks, calculated by triangulation, are expressed in square millimetres.

Results

Table I reports serum levels of total phospholipids, cholesterol and triglycerides. Homozygous thalassaemic patients, compared to normal subjects, showed a reduced level of total phospholipids and total cholesterol ($p < 0.001$)

Analysis of the phospholipid fractions (table II) revealed a decrease in phosphatidylcholine ($p < 0.001$) phosphatidylserine ($p < 0.01$) and sphingomyelin ($p < 0.05$) in homozygotes, compared to normal subjects.

No significant difference was found in triglyceride levels between the groups nor in other lipid levels between heterozygotes and normal subjects.

Gaschromatographic determination of fatty acids in serum lipids (table III fig 1) demonstrates, in thalassaemic homozygous patients as com

Table I Total phospholipids, total cholesterol and triglycerides (mg/dl of plasma) in 20 homozygous for β -thalassaemia, 20 heterozygous and in 20 normal subjects (mean \pm SD and Student's t)

	Homozygotes	Heterozygotes	Normals	p (1-3)
Total phospholipids	285 \pm 63	403 \pm 81	387 \pm 84	< 0.001
Total cholesterol	117 \pm 24	160 \pm 26	173 \pm 34	< 0.001
Triglycerides	110 \pm 39	117 \pm 23	105 \pm 37	NS

NS = Not significant.

Table II Plasma phospholipid fractions (μ g of phospholipidic P/0.1 ml) in 20 homozygous for β -thalassaemia, 20 heterozygous and in 20 normal subjects (mean \pm SD and Student's t)

	Homozygotes	Heterozygotes	Normals	p (1-3)
Phosphatidylserine	18 \pm 11	25 \pm 13	28 \pm 13	< 0.01
Sphingomyelin	18 \pm 7	27 \pm 18	4 \pm 6	< 0.05
Phosphatidylcholine	21 \pm 10	38 \pm 19	34 \pm 12	< 0.001

Table III Fatty acid composition of serum lipids in 20 homozygous for β -thalassaemia, 20 heterozygotes and in 20 normal subjects (mean \pm SD and Student's *t*) values expressed in mm³

Fatty acids	Homozygotes	Heterozygotes	Normals	p (1/3)	p (2/3)
C 14:0	139 \pm 97	65 \pm 26	105 \pm 63	NS	< 0.05
C 16:0	1,038 \pm 775	993 \pm 345	955 \pm 403	NS	NS
C 16:1	149 \pm 118	114 \pm 48	117 \pm 58	NS	NS
C 18:0	273 \pm 176	314 \pm 122	305 \pm 127	NS	NS
C 18:1	1,002 \pm 679	1,098 \pm 605	1,046 \pm 514	NS	NS
C 18:2	452 \pm 388	895 \pm 325	942 \pm 456	< 0.001	NS
C 22:0	36 \pm 33	60 \pm 34	42 \pm 43	NS	NS
C 20:4	81 \pm 44	157 \pm 66	157 \pm 76	< 0.001	NS
C 24:0	55 \pm 43	54 \pm 24	34 \pm 19	NS	< 0.01
After					
C 20:4	85 \pm 87	185 \pm 190	314 \pm 258	< 0.001	< 0.1

NS = Not significant.

Uncertain identification.

Table IV Saturated, monounsaturated, polyunsaturated fatty acid grouping of serum lipids in same subjects (mean \pm SD and Student's *t*) values expressed in mm³

	Homozygotes	Heterozygotes	Controls	p (1/3)
Saturated	308 \pm 210	299 \pm 103	292 \pm 113	NS
Monounsaturated	639 \pm 502	640 \pm 352	581 \pm 290	NS
Polyunsaturated	206 \pm 139	393 \pm 137	471 \pm 205	< 0.001

NS = Not significant.

pared to normal subjects, a highly significant reduction ($p < 0.001$ in all cases) of some long-chain polyunsaturated fatty acids, such as C 18:2, C 20:4 and others following C 20:4. On the whole, there is a significant decrease in polyunsaturated fatty acids ($p < 0.001$) in homozygotic patients compared to normal subjects (table IV fig. 1).

Only minor differences were found in heterozygotes compared to normal subjects. an increase in C 24:0 ($p < 0.01$), a decrease in C 14:0 ($p < 0.05$) and in polyunsaturated fatty acids after C 20:4 ($p < 0.1$).

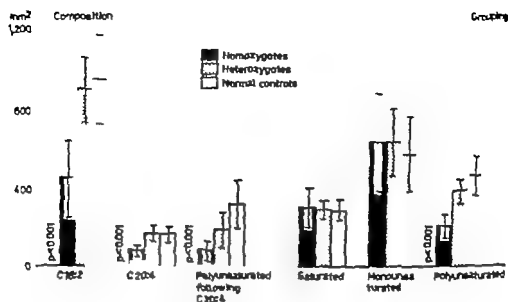


Fig 1 Composition and grouping of serum lipid fatty acids. The values are expressed in mm² (mean \pm SD and Student's t)

Discussion

The data found in the literature regarding serum lipids in homozygotes are rather scarce

PANIZON [22] found a decrease in α -lipoproteins in patients affected by Cooley's disease. CHOREMIS *et al* [5] confirmed this finding and also found an increase of β -lipoproteins and a decrease of total lipids, cholesterol and phospholipids. Both authors attributed these observations to the frequent hepatic damage found in such patients.

The reduction of cholesterol in homozygotes may be due to the low values in their haemoglobin and haematocrit [8-29]. In fact it seems that the value of serum total cholesterol in anaemias of various aetiology is related to the haematocrit [29]. On the other hand, the value of serum total cholesterol does not influence the survival time of erythrocytes, their cholesterol content, nor their osmotic fragility [29].

Our data regarding cholesterol and total phospholipids are in agreement with the above observations [5].

AMERI *et al* [2] found a hypertriglyceridaemia in some patients with thalassemia major and related it to their severe anaemia. The normal

values of serum triglycerides in our groups do not seem to confirm this finding.

We are not aware of other work regarding the serum phospholipid fractions in β -thalassaemic patients. The reduction of all phospholipid fractions found by us in homozygotes could be explained by a generalized deficiency in the synthesis of phospholipids probably due also in this case to hepatic damage.

The serum fatty acid composition showed some changes. In heterozygotes as compared to normal subjects we found an increase of C 24:0 and a decrease of C 14:0 and of some polyunsaturated fatty acids. These findings were negligible and may be attributed to a different diet, especially considering the wide range in age among the heterozygotes group.

In homozygotes compared to heterozygotes and normal subjects, we found a reduction of C 18:2, C 20:4 and of other polyunsaturated fatty acids after C 20:4. These findings are highly significant and are in agreement with the similar data of Olavi *et al.* [21]. This phenomenon, in our opinion, is due to the peroxidation of serum lipids.

In our previous work [20] we found a significant increase in serum malonyldialdehyde (an intermediate product of lipid peroxidation) in the same subjects affected by thalassaemia major studied in the present work. The reduction of the polyunsaturated fatty acids confirms this finding because the polyunsaturated fatty acids are especially susceptible to peroxidation as a result of their methylene groups capable of reacting with oxidative agents. Such affinity increases with the degree of unsaturation [11].

The peroxidation of serum polyunsaturated fatty acids in our β -thalassaemic homozygotes suggest that the cause of lipid peroxidation in β -thalassaemia major need be found not only in the red cells but also in serum. The cause might be looked for in our opinion, in the iron overload present in homozygotes.

In fact, iron in a bivalent form is a powerful oxidant: it catalyzes the generation of free radicals capable in turn of reacting with polyunsaturated fatty acids: the latter become oxidized, thus giving rise to additional free radicals [12]. This series of reactions is normally avoided since circulating iron in trivalent form gets bound by the plasma transferrin [1].

In β -thalassaemic homozygotes, serum iron levels are elevated and transferrin, being almost completely saturated [13], cannot exert its antioxidant action due to its iron-binding capacity [19].

In β -thalassaemia, moreover serum levels of haemoglobin suitable for reducing ferric iron to ferrous form are increased [18]. These findings

might warrant the hypothesis that red cells and plasma lipid peroxidation in β thalassaemia major is at least partly connected with iron overload.

That assumption could explain the decrease of plasma vitamin E found by some authors [14 23 30] in β -thalassaemic homozygotes, because this vitamin is apt to bind free radicals by becoming oxidized to paraquinone [28]

Further studies of ferrous/ferric iron relationship in serum and of oxidation derivatives of tocopherols are needed to clarify the above problem.

References

- 1 Aiken, P. The binding and release of iron by transferrin, in BERGMAN, CERAMI, PETERSON and GRAZIANO Iron metabolism and thalassaemia, pp. 88-95 (Liss, New York 1976)
- 2 AMERI, M. R., ALBOUYEH, M. and ZIAI, M. Hypertriglyceridaemia in homozygous beta-thalassaemia. *J. Pediatr.* 87 1002-1003 (1975)
- 3 BADDELEY, E. S., FOŁ, P. P. and ZAK, B. Microdetermination of inorganic phosphate, phospholipids and total phosphate in biologic materials. *Clin. Chem.* 13 326-332 (1967).
- 4 BRADGON J. H. Total lipids in serum in SUNDERMAN and SUNDERMAN jr Lipids and steroid hormones in clinical medicine pp 9-11 (Lippincott, Philadelphia 1960)
- 5 CHORINIS, C., KYRIAKIDIS, V. and PAPADAKIS, E. Studies on the blood lipids and lipoproteins in thalassaemia and sickle cell anaemia. *J. clin. Path.* 14 361-364 (1961)
- 6 DODGE, J. T. and PHILLIPS, G. B. Autoxidation as a cause of altered lipid distribution in extracts from human red cells. *J. Lipid Res.* 7 387-394 (1966).
- 7 DODGE, J. T. and PHILLIPS, G. B. Composition of phospholipids and of phospholipid fatty acids and aldehydes in human red cells. *J. Lipid Res.* 8 667-675 (1967).
- 8 FERIAS, P., STAMATOYANNOPoulos, G. and KEYS, A. Serum cholesterol and thalassaemia trait. *Lancet* i. 1182-1183 (1963).
- 9 FLETCHER, M. S. A colorimetric method for estimating serum triglycerides. *Clinica chim. Acta* 22 393-397 (1968).
- 10 GIARDINI, G., CASTRO, M., and MAGGIORI, G. Increased susceptibility of red blood cell lipids to autooxidation in thalassaemia major. *Lancet* i. 33 (1974).
- 11 GRAZIANO, J. H., MILLER, D. R., GRADY R. W. and CERAMI, A. Inhibition of membrane peroxidation in thalassaemic erythrocytes by 2,3-dihydroxybenzoic acid. *Br J Haemat.* 32 351-356 (1976).
- 12 GRAZIANO J. H. Potential usefulness of free radical scavengers in iron overload in BERGMAN, CERAMI, PETERSON and GRAZIANO Iron metabolism and thalassaemia, pp. 135-143 (Liss, New York 1976).

- 13 HERSKO, C. Pathogenesis and management of iron overload in thalassemia. *Haematologica* 60: 241-248 (1975).
- 14 HYMAN, C. B., LAMONTE, B., ALPIN SLATER, R., KOZAK, L., WEITZMAN, J. and ORTEGA, J. A. *dl*- α -Tocopherol, iron and lipofuscin in thalassemia. *Ann. N.Y. Acad. Sci.* 232, 211-220 (1974).
- 15 JACOB, H. S. and LUX, S. E., IV. Degradation of membrane phospholipids and thiols in peroxide haemolysis. Studies in vitamin E deficiency. *Blood* 32, 549-578 (1968).
- 16 JAMES, A. T., LOVELOCK, J. E., WESS, J. and TROTTER, W. R. The fatty acids of the blood in coronary artery disease. *Lancet* i, 705-708 (1957).
- 17 MAIORANI, G., CASTRO, M., DONFRANCESCO, A., SPANO, B., and GIARDINO, O. Haemolysis and erythrocyte lipids in thalassemia major. *Acta haemat.* 57, 207-213 (1974).
- 18 MANNING, C. E. Rancidity of the red cell. *Am. J. med. Sci.* 255, 341 (1968).
- 19 MCDELL, C. B., STOCKS, J. and DORMANDY T. L. Vitamin E in thalassemia. *Br. med. J.* iii, 259 (1974).
- 20 MURIELA, F., MARTINO, F., MARCHANDI, O., SPANO, B., GIARDINO, O. - MAIORANI, G. Perossidazione lipidica ed emolisi nella β -talassemia. Comunicazione al I Congresso Nazionale della Società Italiana per lo studio degli errori metabolici congeniti. Sanremo 1976.
- 21 OLIVE, O., GEMOVA, R., MURIELA, C., MARTINO, E. Analisi gascromatografica degli acidi grassi plasmatici ed eritrocitari in soggetti normali ed in pazienti affetti da Morbo di Cooley. *Haematologica* 55, 334-342 (1968).
- 22 PANZONI, F. Il lipidogramma elettroforetico nella Malattia di Cooley. *Acta paediat. Lat.* 10, 730-753 (1957).
- 23 RACHOWITZ, E. A., LUTEN, B. H. and SMOLEY, S. B. Lipid membrane peroxidation in beta-thalassemia major. *Blood* 47, 495-505 (1976).
- 24 ROSCHILLI, P., BEZANT, E., and ORMER, W. Enzymatic determination of total cholesterol in serum. *Z. klin. Chem. klin. Biochem.* 12, 226-229 (1974).
- 25 ROSCHILLI, H. The detection of phospholipids on chromatograms. *J. Chromatog.* 2, 487-489 (1959).
- 26 SKIPPIN, V. C., PETERSON, R. F. and BARCLAY, M. Quantitative analysis of phospholipids by thin-layer chromatography. *Biochem. J.* 90, 374-377 (1964).
- 27 STOCKS, J., KEMP, M., and DORMANDY T. L. Increased susceptibility of red blood cell lipids to autoxidation in haemolytic states. *Lancet* i, 266-269 (1971).
- 28 TAPPEL, A. L. Vitamin E and free radical peroxidation of lipids. *Ann. N.Y. Acad. Sci.* 703, 12-28 (1972).
- 29 WESTERMAN, M. Hypocholsterolaemia and anaemia. *Br. J. Haemat.* 31, 87-94 (1971).
- 30 ZANNO, MARCOLA, L., TEOTIZATOU, F., DIMOSSI SVOLAKI, K., KATERILLOS, C. H., KAVALLARI, M., and MATRANGOLIS, N. Serum vitamin E levels with beta-thalassemia major. Preliminary report. *Br. J. Haemat.* 26, 193-199 (1974).

'Classic' and 'Acute' Myelofibrosis

A Retrospective Study

R A ROVER, N H MULDER and H O NIEWEG

Division of Haematology Department of Medicine, University of Groningen,
Groningen

Key Words. Acute myelofibrosis Classic myelofibrosis LDH in myelofibrosis
Prognosis of myelofibrosis Reticulocytes in myelofibrosis Splenomegaly in
myelofibrosis SRE in myelofibrosis Uric acid in myelofibrosis

Abstracts In a retrospective study of 38 patients with histologically proven myelofibrosis, 5 parameters (recorded on the first admission) were investigated as to their usefulness to predict the course of the disease. In 9 patients the development of 'acute' myelofibrosis could be predicted by the finding of pancytopenia, low reticulocyte counts (median 20,500/ μ l), extremely elevated SRE (median 125 mm) and normal serum levels of LDH and uric acid on the first admission. In 28 patients the development of classic fibrosis with splenomegaly could be predicted by the finding of high reticulocyte counts (median 133,200/ μ l) and increased levels of serum LDH (median 547 U) and uric acid (median 8 mg/dl in males and 6.8 mg/dl in females) on the first admission, even when splenomegaly was initially absent. The relationship between classic and acute myelofibrosis and the significance of the mentioned parameters is discussed.

Classic myelofibrosis is a chronic disorder of blood formation. Its characteristic features are considered to be an increase of fibrous tissue and clusters of megakaryocytes in the bone marrow extramedullary hematopoiesis and leuco-erythroblastosis in the peripheral blood. Progressive splenomegaly is the most consistent finding on physical examination and the clinical course is usually chronic, slowly progressive, though ultimately fatal.

It is important to recognize a variant of this syndrome described by WOOD and ANDREWS [24] and LEWIS and SZUR [11] since the clinical

course of this variant is often rapidly fatal. The term acute myelofibrosis has been suggested by BERGSMAN and VAN SLICK [1]. Absence of a palpable spleen is typical of acute myelofibrosis.

However splenomegaly may also be absent at an early stage of classic myelofibrosis. So if a patient presents with the above mentioned criteria for myelofibrosis without splenomegaly the question arises whether the case will prove to be one of classic myelofibrosis (characterized by a mostly chronic course and the development of splenomegaly) or one of acute myelofibrosis (characterized by a shorter survival while the spleen becomes so enlarged as to be palpable).

In this retrospective study we have tried to find parameters, present at an early stage of the disease, which might predict its ultimate course.

Patients and Methods

Patients

38 patients with idiopathic myelofibrosis were studied retrospectively. 33 patients were initially admitted at some time between January 1st, 1967 and May 1st, 1977. In addition, we evaluated 5 patients in whom the diagnosis had been established before this period. These latter cases were not included in the survival curves.

Pathology

In all patients bone marrow biopsy specimens were obtained from the posterior iliac crest using either the Westerman or the Jamshidi needle. The specimens were examined by A. AARDE MD and R. EUSTACHE MD (Department of Pathology). Essential for the diagnosis were the presence in the biopsy specimen of an increase in fibrous tissue [10] (collagen and/or reticulum) and clusters of atypical megakaryocytes or megakaryoblasts [22]. Leuco-erythroblastosis and teardrop erythrocytes in May-Grimwald-Giemsa stained smears of peripheral blood were also mandatory. Additional findings [23] included: histological proof of extramedullary haemopoiesis in spleen, liver or lymph node, bizarre or giant thrombocytes in the peripheral blood smear, evolution from polycythemia vera, and finally evidence of osteosclerotic rosettes on X-ray examination.

Prognostic Parameters

(a) Peripheral pancytopenia was diagnosed when haemoglobin was below 14 g/dl, polymorphonuclear leucocytes less than $4,000/\mu\text{l}$ and platelets less than $120,000/\mu\text{l}$. The haemoglobin was measured by an optical density method as cyanmethaemoglobin [18]. Counts of peripheral cells were made electronically as routine and by the classical microscopic method when leucocytes were less than $4,000/\mu\text{l}$ or thrombocytes less than $80,000/\mu\text{l}$. The haematocrit was deduced from measurements of the red cell count and MCV on the Coulter 3 counter [18]. Before 1973, the haematocrit was determined by microcentrifuge method.

'Classic' and 'Acute' Myelofibrosis

A Retrospective Study

R. A. ROYER, N. H. MULDER and H. O. NIEWEG

Division of Haematology Department of Medicine, University of Groningen,
Groningen

Key Words: Acute myelofibrosis Classic myelofibrosis LDH in myelofibrosis
Prognosis of myelofibrosis Reticulocytes in myelofibrosis Splenomegaly in
myelofibrosis SRB in myelofibrosis Uric acid in myelofibrosis

Abstracts. In a retrospective study of 38 patients with histologically proven myelofibrosis, 5 parameters (recorded on the first admission) were investigated as to their usefulness to predict the course of the disease. In 9 patients the development of 'acute' myelofibrosis could be predicted by the finding of pancytopenia, low reticulocyte counts (median 20,500/ μ l), extremely elevated SRB (median 125 mm) and normal serum levels of LDH and uric acid on the first admission. In 28 patients the development of classic fibrosis with splenomegaly could be predicted by the finding of high reticulocyte counts (median 133,200/ μ l) and increased levels of serum LDH (median 547 U) and uric acid (median 8 mg/dl in males and 6.8 mg/dl in females) on the first admission, even when splenomegaly was initially absent. The relationship between classic and acute myelofibrosis and the significance of the mentioned parameters is discussed.

Classic myelofibrosis is a chronic disorder of blood formation. Its characteristic features are considered to be an increase of fibrous tissue and clusters of megakaryocytes in the bone marrow, extramedullary hemopoiesis and leuco-erythroblastosis in the peripheral blood. Progressive splenomegaly is the most consistent finding on physical examination and the clinical course is usually chronic, slowly progressive, though ultimately fatal.

It is important to recognize a variant of this syndrome described by WOOD and ANDREWS [24] and LEWIS and SZUR [11] since the clinical

course of this variant is often rapidly fatal. The term acute myelofibrosis has been suggested by BERGSMAN and VAN SLICK [1]. Absence of a palpable spleen is typical of acute myelofibrosis.

However splenomegaly may also be absent at an early stage of classic myelofibrosis. So if a patient presents with the above-mentioned criteria for myelofibrosis without splenomegaly the question arises whether the case will prove to be one of 'classic' myelofibrosis (characterized by a mostly chronic course and the development of splenomegaly) or one of acute myelofibrosis (characterized by a shorter survival while the spleen becomes so enlarged as to be palpable).

In this retrospective study we have tried to find parameters, present at an early stage of the disease, which might predict its ultimate course.

Patients and Methods

Patients

38 patients with idiopathic myelofibrosis were studied retrospectively. 33 patients were initially admitted at some time between January 1st, 1967 and May 1st, 1977. In addition, we evaluated 5 patients in whom the diagnosis had been established before this period. These latter cases were not included in the survival curves.

Pathology

In all patients bone marrow biopsy specimens were obtained from the posterior iliac crest using either the Westerman or the Jamshidi needle. The specimens were examined by A. AARWIS MD and R. ENKHOVEN MD (Department of Pathology). Essential for the diagnosis were the presence in the biopsy specimen of an increase in fibrous tissue [10] (collagen and/or reticulin) and clusters of atypical megakaryocytes or megakaryoblasts [22]. Leuco-erythroblastosis and teardrop erythrocytes in May-Grünwald-Giemsa stained smears of peripheral blood were also mandatory. Additive findings [23] included histological proof of extramedullary haemopoiesis in spleen, liver or lymph node, bizarre or giant thrombocytes in the peripheral blood smear, evolution from polycythaemia vera, and finally evidence of osteosclerosis on X-ray examination.

Prognostic Parameters

(a) Peripheral procytopenia was diagnosed when haemoglobin was below 12 g/dl, polymorphonuclear leucocytes less than $\cdot,000/\mu\text{l}$ and platelets less than $120,000/\mu\text{l}$. The haemoglobin was measured by an optical density method as cyanmethaemoglobin [18]. Counts of peripheral cells were made electronically as routine and by the classical microscopic method when leucocytes were less than $2,000/\mu\text{l}$ or thrombocytes less than $80,000/\mu\text{l}$. The haematocrit was deduced from measurements of the red cell count and MCV on the Coulter S counter [18]. Before 1975, the haematocrit was determined by microcentrifuge method.

(b) The number of reticulocytes per 1000 red blood cells was counted in brilliant cresyl blue-stained smears. The number per microlitre blood was calculated from this figure and the electronically determined red cell count [6]. The normal reticulocyte concentration is about 60,000/ μ l. The reticulocyte count was not used as a parameter in case of overt haemolysis, recent blood transfusions or recently started therapy with anti-anaemic compounds.

(c) The 1-hour sedimentation rate of erythrocytes (SRE) was determined according to Westergren. It was only used as a parameter in 11 patients with comparable packed red cell volumes. No clinical signs of infection or recent trauma had been found in these 18 patients.

(d) Serum lactic dehydrogenase activity (LDH) was determined according to KROO [9] and since 1975 according to MORGENTHAU *et al.* [13]. With the latter method a multiplying factor of 1.489 was used to obtain comparable values. Normal range: 170–350 U. Serum LDH was only taken into account after the exclusion of overt haemolysis, vitamin B₁₂ or folic acid deficiency and finally myocard, muscle or liver disease. LDH isoenzymes were estimated in 5 patients [25].

(e) Serum uric acid was determined according to the method of SOMMER-SNOELS [19] modified by MURRAY and ORTIZOZA [14]. Normal range for men \approx 1–7.6 mg/dl, women 1.8–6.5 mg/dl. Serum uric acid was only taken into account when renal insufficiency or the simultaneous use of thiazide diuretics, uricosuric or cytostatic drugs were excluded.

The above-mentioned parameters were recorded on the first admission of the patient when the diagnosis of myelofibrosis was made. Each parameter was expressed as a figure representing the median value from 3 determinations.

The patients were examined for splenomegaly by manual palpation. In addition a ⁹⁹Tc colloid scintigram of the liver and spleen was routinely made in the Central Isotope Laboratory by M. G. WOLBRING PhD, H. BEERHUIS, PhD and D. A. PICKS, MD (gamma-camera, 3m C).

Survival curves. Survival curves were constructed for 33 patients according to CUTLER and EDEKER [3]. Death was attributed to myelofibrosis when caused by thrombocytopenic haemorrhage, granulocytopenic infection or anaemia.

Statistics. Statistical analysis of the parameters mentioned was estimated by Wilcoxon's rank sum test. 2p values less than 0.05 were considered significant. The survival curves were analysed according to GERMAN'S [4] modification of the Wilcoxon test.

Results

In this series 9 patients did not develop splenomegaly while 29 others did. The median values for LDH, uric acid, reticulocyte count and SRE in these two groups (respectively acute and classic myelofibrosis) are shown in table I. Serum LDH, uric acid and reticulocyte count are significantly higher on the first admission in patients who will follow the clinical course of classic myelofibrosis. The 1-hour blood sedimentation rate is

Table I

Parameters	Classic myelofibrosis	Acute myelofibrosis	S
Reticulocyte count	n 29 MV 133,200/ μ l SD 78,818	9 20,500/ μ l 14,513	2 p < 0.01
Serum LDH	n 28 MV 547 U SD 402	9 306 U 87	2 p < 0.01
Serum uric acid	22 MV male 8 mg/dl female 6.8 mg/dl SD 2.49	5 male 4.5 mg/dl female 3.8 mg/dl 1.09	2 p < 0.01
SRE	n 9 MV 60 mm SD 23	9 125 mm 45	0.02 < 2 p < 0.05

MV = Median value n = number of patients S = significance of difference in the parameters mentioned between the group with classic myelofibrosis and the group with acute myelofibrosis. The parameters were recorded on the first admission of each patient. Myelofibrosis was qualified as 'classic' in patients in whom splenomegaly was found at some stage of the disease and as 'acute' in patients who died of myelofibrosis while palpable spleen had not been found at any stage of the disease.

significantly higher in the group with acute myelofibrosis. It is shown in table II that haemoglobin level, granulocyte and thrombocyte counts are significantly lower in the group with acute myelofibrosis. Pancytopenia on the first admission is more frequent in this group (table III)

In no patient with acute myelofibrosis an increase of reticulocyte count or of serum uric acid was found, but there was a slight elevation of LDH value in 2 of 9 patients with acute myelofibrosis. When any two of the above-mentioned parameters were applied, no overlap between the 'acute' and the 'classic' group occurred.

LDH isoenzymes have been determined in 5 cases with classic myelofibrosis. Each time an elevation of isoenzymes II and III was found. 3 patients out of the group of 29 who developed classic myelofibrosis presented initially without splenomegaly. Retrospectively it would have been possible to predict the course of their disease at the moment of presentation relying on the above-mentioned parameters.

Table II Median values (MV) for levels of haemoglobin and for counts of peripheral granulocytes and thrombocytes on the first admission of 29 patients with classic and 9 patients with acute myelofibrosis

	Classic myelofibrosis, MV	Acute myelofibrosis, MV	S
Haemoglobin	10 g/dl	6.9 g/dl	p < 0.01
Granulocytes	7,568 μ l	704 μ l	2 p < 0.01
Thrombocytes	190,000 μ l	37 000 μ l	2 p < 0.01

Statistical significance of difference between these groups (S) The criteria used to make these groups are provided in the legend to table I

Table III Number of patients presenting on their first admission with anaemia, granulocytopenia, thrombocytopenia or pancytopenia in classic myelofibrosis (total 29 patients) and acute myelofibrosis (total 9 patients)

	Classic myelofibrosis	Acute myelofibrosis
Anaemia	17	8
Granulocytopenia	7	8
Thrombocytopenia	9	8
Pancytopenia	3	8

The criteria used to make these groups are provided in the legend to table I

No correlation could be found between the severity of splenomegaly and these parameters nor between these parameters individually. The prognosis of acute and chronic myelofibrosis is visualized in the survival curve (fig. 1). It can be seen that the cumulative survival of those patients who did not develop splenomegaly and who presented with pancytopenia, increased SRE, low values of serum uric acid or LDH and low reticulocyte count, is significantly shorter than that of patients from the other group with classic myelofibrosis. Survival in 2 of the 3 patients with classic myelofibrosis who presented with pancytopenia, was better than in the group with acute myelofibrosis. 1 of these patients survived for 2 years, but another 1 survived for more than 6 years while the third patient is still alive, 3 1/2 years since the diagnosis of myelofibrosis was made.

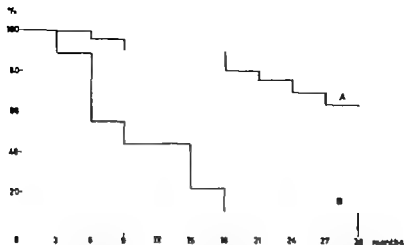


Fig 1 Cumulative survival of 25 patients with myelofibrosis (A) and 9 patients with acute myelofibrosis (B). Survival in group B is significantly shorter after 6 months period ($p < 0.01$). On the first admission of patients from group B pancytopenia was commonly found, the SRE strikingly elevated and reticulocyte counts, serum LDH and uric acid lower than on the first admission of patients from group A. The criteria used to make these groups are provided in the legend to table 1. The diagnosis myelofibrosis was made at 0 months.

Discussion

It might be assumed [21] that the time may be too short for the development of splenic enlargement in acute myelofibrosis. However in our series the longest survivor of acute myelofibrosis died 2 1/2 years after diagnosis, while in all patients with classic myelofibrosis splenomegaly had developed within the first year after diagnosis. Furthermore, splenomegaly can sometimes develop in a very short time in classic myelofibrosis. It must be emphasized that acute myelofibrosis is different from a malignant transformation in classic myelofibrosis. Blast cells were never conspicuous in the peripheral blood of our patients with acute myelofibrosis (less than 5% of the leucocyte count). This is in accordance with the description by LEWIS and SZUR [11] who reported that on presentation of their patients less than 7% blasts were found in the peripheral blood. PATEL *et al* [15] recently described a case history of rapidly fatal myelofibrosis without splenomegaly but presenting with acute myeloblastic leu-

Table II Median values (MV) for levels of haemoglobin and for counts of peripheral granulocytes and thrombocytes on the first admission of 29 patients with classic and 9 patients with acute myelofibrosis

	Classic myelofibrosis, MV	Acute myelofibrosis, MV	S
Haemoglobin	10 g/dl	6.9 g/dl	2 p < 0.01
Granulocytes	7,568 μ l	704 μ l	2 p < 0.01
Thrombocytes	190 000 μ l	37,000 μ l	2 p < 0.01

Statistical significance of difference between these groups (S). The criteria used to make these groups are provided in the legend to table I

Table III Number of patients presenting on their first admission with anaemia, granulocytopenia, thrombocytopenia or pancytopenia in classic myelofibrosis (total 29 patients) and acute myelofibrosis (total 9 patients)

	Classic myelofibrosis	Acute myelofibrosis
Anaemia	17	8
Granulocytopenia	7	8
Thrombocytopenia	9	8
Pancytopenia	3	8

The criteria used to make these groups are provided in the legend to table I

No correlation could be found between the severity of splenomegaly and these parameters nor between these parameters individually. The prognosis of acute and chronic myelofibrosis is visualized in the survival curve (fig. 1). It can be seen that the cumulative survival of those patients who did not develop splenomegaly and who presented with pancytopenia, increased SRE, low values of serum uric acid or LDH and low reticulocyte count, is significantly shorter than that of patients from the other group with classic myelofibrosis. Survival in 2 of the 3 patients with classic myelofibrosis who presented with pancytopenia, was better than in the group with acute myelofibrosis. 1 of these patients survived for 2 years, but another 1 survived for more than 6 years while the third patient is still alive, 3 $\frac{1}{2}$ years since the diagnosis of myelofibrosis was made.

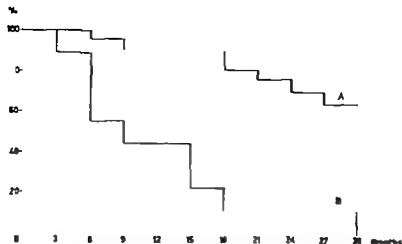


Fig 1 Cumulative survival of 25 patients with myelofibrosis (A) and 9 patients with acute myelofibrosis (B). Survival in group B is significantly shorter after 6 months period ($p < 0.01$). On the first admission of patients from group B pancytopenia is commonly found, the SRE strikingly elevated and reticulocyte counts, serum LDH and uric acid lower than on the first admission of patients from group A. The criteria used to make these groups are provided in the legend to table 1. The diagnosis myelofibrosis was made at 0 months.

Discussion

It might be assumed [21] that the time may be too short for the development of splenic enlargement in acute myelofibrosis. However in our series the longest survivor of acute myelofibrosis died 2 / years after diagnosis, while in all patients with classic myelofibrosis splenomegaly had developed within the first year after diagnosis. Furthermore, splenomegaly can sometimes develop in a very short time in classic myelofibrosis. It must be emphasized that acute myelofibrosis is different from a malignant transformation in classic myelofibrosis. Blast cells were never conspicuous in the peripheral blood of our patients with acute myelofibrosis (less than 5% of the leucocyte count). This is in accordance with the description by Lewis and Szur [11] who reported that on presentation of their patients less than 7% blasts were found in the peripheral blood. PATEL *et al* [15] recently described a case history of rapidly fatal myelofibrosis without splenomegaly but presenting with acute myeloblastic leu-

laemia. The authors concluded that this could have been a case either of acute myelofibrosis with blastic proliferation or of acute leukaemia with preparative myelofibrosis [17]. It is obvious that the mutual relationship between these variants is still to be clarified.

The explanation of the increased levels of serum LDH and uric acid in classic myelofibrosis is not clear. Elevation of LDH isoenzymes II and III suggests that breakdown of red blood cells cannot be the only explanation. The finding of high levels of LDH in classic myelofibrosis was also described by HOFFBRAND *et al* [8]. In polycythaemia vera a condition with an increased production of red blood cells, these authors have found normal LDH levels. Serum uric acid on the other hand is frequently elevated in polycythaemia vera [5]. It has been suggested by HICKLING [7] that serum uric acid levels in myelofibrosis correlate with the number of megakaryocytes in the bone marrow.

Peripheral pancytopenia on the first admission was rarely found in the group with classic myelofibrosis but it has been present in most patients with acute myelofibrosis. Causes of death in myelofibrosis are related to pancytopenia. Pancytopenia deteriorates rapidly in patients with acute myelofibrosis, which might explain the short survival in this group. It has been stated that reticulocytosis in myelofibrosis reflects the escape of many immature erythrocytes into the peripheral blood (shift reticulocytosis) [16]. The occurrence of peripheral leuco-erythroblastosis is also explained by this shift phenomenon. As leuco-erythroblastosis is more marked in classic myelofibrosis [11] it may be concluded that more immature cells escape to the peripheral blood in classic myelofibrosis as compared to acute myelofibrosis. Absence of reticulocytosis in acute myelofibrosis may be related to this quantitative difference in shift. It is possible that reticulocytosis in myelofibrosis is not only caused by shift. WARD and BLOCK [23] for instance found no correlation between the degree of reticulocytosis and the degree of normoblastaemia in myelofibrosis. It might be suggested that reticulocytosis in myelofibrosis is also due to some other process, for instance increase of erythropoietic activity. It has been demonstrated by ferrokinetic studies that the number of red cell precursors is increased in classic myelofibrosis [20] but not so in acute myelofibrosis [2, 12]. So absence of reticulocytosis in acute myelofibrosis might be due to decreased erythropoietic activity as well as to a lesser degree of shift. Both factors may be related to the extent of extramedullary haemopoiesis.

As far as SRE, the last of the mentioned parameters, is concerned its

increased levels seem to coincide with hyperglobulinaemia in our cases of acute myelofibrosis. As yet, the explanation of this phenomenon is unclear. Further analysis of this problem might possibly lead to a clue in respect to the (auto-immune?) cause of this variant of myelofibrosis. It can be concluded from our data that the occurrence of pancytopenia (table II III) as well as the values for SRE, reticulocyte count, serum uric acid and LDH (table I) on the first admission of a patient with myelofibrosis, are of prognostic significance as to the chance for survival in such a patient (fig. 1).

References

- 1 BERGMAN K. L. and V. N. SLICK, E. J. Acute myelofibrosis: An accelerated variant of agnogenic myeloid metaplasia. *Ann. Intern. Med.* 74: 232-235 (1971).
- 2 BAÏRE, J., CASTRO-MALEPOMA, H., BAÏRE, J. F. et BERNAUD, J. Les myelofibroses aiguës ou subaiguës. *Nouv. Revue fr. Hémat.* 16: 3-22 (1976).
- 3 CUTLER, S. J. and EDWARDS, F. Maximum utilization of the life table method in analyzing survival. *J. chron. Dis.* 8: 699-712 (1956).
- 4 GERAN, E. A. A generalized Wilcoxon test for comparing arbitrarily singly-censored samples. *Biometrika* 52: 203-233 (1965).
- 5 GILBERT, H. S. Definition, clinical features and diagnosis of PV. *Clin. Haemat.* 4: 263-291 (1975).
- 6 HARRIS, J. W. and KELLERMEYER, R. W. The red cell, p. 313 (Harvard University Press, Cambridge 1970).
- 7 HICKLING, R. A. Leukemia and related conditions and the blood uric-acid. *Lancet* 175-178 (1958).
- 8 HOFFERLAND, A. V., KREMOUCHEVSKY, S., BUTTERWORTH, P. J. and MOLLIN, D. H. Serum lactic dehydrogenase activity and folate deficiency in myeloclerosis and other hematological diseases. *Br. med. J.* 1: 577-581 (1961).
- 9 KING, J. A routine method for the estimation of lactic dehydrogenase activity. *J. med. Lab. Tech.* 16: 265 (1959).
- 10 LEONERT, K., NAGAI, K. and SCHWABER, E. W. Patho-anatomical features of the bone marrow. *Clin. Haemat.* 4: 331-351 (1975).
- 11 LEWIS, S. M. and SKIN, L. Malignant myeloclerosis. *Br. med. J.* 4: 472-477 (1963).
- 12 MCVIE, J. G., MACHMUEHL, A. C. and RAMSA, H. M. Progression of polycythemia vera to malignant myelofibrosis and reticulum cell sarcoma. *Scand. J. Haematol.* 13: 61-63 (1974).
- 13 MORGENTHAU, S., FLOW, R., KESLER, G. and ALLEN, B. Automated determination of NAD-coupled enzymes, determination of lactic dehydrogenase. *Analyt. Biochem.* 13: 149-161 (1965).
- 14 MURPHY, W. A. and ORTIZOZA, C. Automatic determination of uric acid by the hydroxylamine method. *Tech. Bull. Reg. med. Tech.* 30: 21-25 (1966).

laemia. The authors concluded that this could have been a case either of acute myelofibrosis with blastic proliferation or of acute leukaemia with preparative myelofibrosis [17]. It is obvious that the mutual relationship between these variants is still to be clarified.

The explanation of the increased levels of serum LDH and uric acid in classic myelofibrosis is not clear. Elevation of LDH isoenzymes II and III suggests that breakdown of red blood cells cannot be the only explanation. The finding of high levels of LDH in classic myelofibrosis was also described by HOFFBRAND *et al* [8]. In polycythaemia vera, a condition with an increased production of red blood cells, these authors have found normal LDH levels. Serum uric acid on the other hand is frequently elevated in polycythaemia vera [5]. It has been suggested by HICKLING [7] that serum uric acid levels in myelofibrosis correlate with the number of megakaryocytes in the bone marrow.

Peripheral pancytopenia on the first admission was rarely found in the group with classic myelofibrosis, but it has been present in most patients with acute myelofibrosis. Causes of death in myelofibrosis are related to pancytopenia. Pancytopenia deteriorates rapidly in patients with acute myelofibrosis which might explain the short survival in this group. It has been stated that reticulocytosis in myelofibrosis reflects the escape of many immature erythrocytes into the peripheral blood (shift reticulocytosis) [16]. The occurrence of peripheral leuco-erythroblastosis is also explained by this shift phenomenon. As leuco-erythroblastosis is more marked in classic myelofibrosis [11] it may be concluded that more immature cells escape to the peripheral blood in classic myelofibrosis as compared to acute myelofibrosis. Absence of reticulocytosis in acute myelofibrosis may be related to this quantitative difference in shift. It is possible that reticulocytosis in myelofibrosis is not only caused by shift. WARD and BLOCK [23] for instance found no correlation between the degree of reticulocytosis and the degree of normoblastaemia in myelofibrosis. It might be suggested that reticulocytosis in myelofibrosis is also due to some other process, for instance increase of erythropoietic activity. It has been demonstrated by ferrokinetic studies that the number of red cell precursors is increased in classic myelofibrosis [20] but not so in acute myelofibrosis [2, 12]. So absence of reticulocytosis in acute myelofibrosis might be due to decreased erythropoietic activity as well as to a lesser degree of shift. Both factors may be related to the extent of extramedullary haemopoiesis.

As far as SRE, the last of the mentioned parameters, is concerned its

Peripheral Cryoglobulinemic Neuropathy in a Patient with Gaucher's Disease

D. BENJAMIN, D. DOUVER, A. I. PICK, M. ZER, M. DENTEMAN and J. PINKHAS

Departments of Medicine D and Surgery A and the Section of Clinical Immunology Beilinson Medical Center Tel Aviv University Medical School, Petah Tikva

Key Words. Cryoglobulinaemia. Gaucher's disease. Neuropathy cryoglobulinemic

Abstract. Cryoglobulinaemia with peripheral cryoglobulinemic neuropathy is reported in a patient with Gaucher's disease. To the best of our knowledge, similar relationship has not been previously described. The question whether the cryoglobulinemic neuropathy in this patient is related to Gaucher's disease, or is mere coincidence, is discussed.

Peripheral neuropathy is known to occur in 7-17% of patients with cryoglobulinemia [4, 16] either essential [17-21] or secondary to diseases such as lymphoma [3, 4], Waldenström's macroglobulinemia [7], periarteritis nodosa [11], liver cirrhosis [15] etc.

We presently report a patient with Gaucher's disease and cryoglobulinemia with peripheral neuropathy, an association which, to the best of our knowledge was hitherto not described.

Case Report

A 70-year-old housewife in whom Gaucher's disease was diagnosed in 1940, was hospitalized in October 1975 due to weakness, arthralgia and purpura.

In 1961 she was splenectomized because of massive enlargement of the spleen with abdominal discomfort and marked thrombocytopenia. In 1962 she underwent left hemicolectomy because of adenocarcinoma of the transverse colon. According to her family physician, 1 month prior to her present admission she started to complain of vague abdominal pains and purpura in both legs. An upper gastrointestinal

- 15 PATEL, A. R. SHAM, P. C., and RHEE, H. L. Acute myelofibrosis and acute myeloblastic leukaemia. *Br med. J* // 123^o (1976)
- 16 RAPAPORT S. I. Introduction to haematology (Harper & Row Hagerstown 1971).
- 17 SANERKIN N. G. Stromal changes in leukaemic and related bone marrow proliferations. *J clin Path.* 17 541-547 (1964)
- 18 SHARP A. A. and BALLARD C. D.. An evaluation of the Coulter S counter *J clin. Path.* 23 327-335 (1970)
- 19 SOMRINO, SMOLA, M. A sensitive method for the measurement of serum uric acid using hydroxylamine. *J Lab clin. Med.* 65 665-668 (1965)
- 20 SZUR, L. and LEWIS, S. M. Iron kinetics. *Clin Haemat* 4 407-425 (1975)
- 21 VAN SLICK, E. J. WYRS, L. and DULLY M.. Chromosomal evidence for the secondary role of fibroblastic proliferation in acute myelofibrosis. *Blood* 36 729-735 (1970)
- 22 WAITZ, R. MAYER, S. et MAYER, G. Splénomégalie myéloïde et autres formes d'ostéomyélosclérose *Sang* 3 1-16 (1963)
- 23 WARD, H. P. and BLOCK, M. H. The natural history of agnogenic myeloid metaplasia (AMM) and a critical evaluation of its relationship with the myeloproliferative syndrome. *Medicine* 50 357-420 (1971)
- 24 WOOD, E. E. and ANDREWS, C. T. Subacute myeloclerosis, report of three cases. *Lancet* // 739-748 (1949)
- 25 ZOEMAO, H. A. Determination and diagnostic significance of lactate dehydrogenase isoenzymes (Van Gorcum, Assen 1967).

Peripheral Cryoglobulinemic Neuropathy in a Patient with Gaucher's Disease

D. BENJAMIN, D. DOVER, A. L. PICK, M. ZER, M. DINTSEMAN and J. PINKHAS

Departments of Medicine 'D' and Surgery 'A' and the Section of Clinical Immunology Beilinson Medical Center Tel Aviv University Medical School, Petah Tikva

Key Words. Cryoglobulinemia Gaucher' disease Neuropathy cryoglobulinemic

Abstract. Cryoglobulinemia with peripheral cryoglobulinemic neuropathy is reported in a patient with Gaucher' disease. To the best of our knowledge, similar relationship has not been previously described. The question whether the cryoglobulinemic neuropathy in this patient is related to Gaucher's disease, or is more coincidental, is discussed.

Peripheral neuropathy is known to occur in 7-17% of patients with cryoglobulinemia [4, 16] either essential [17, 21] or secondary to diseases such as lymphoma [3, 4], Waldenström's macroglobulinemia [7], periarteritis nodosa [11], liver cirrhosis [15] etc.

We presently report a patient with Gaucher's disease and cryoglobulinemia with peripheral neuropathy, an association which, to the best of our knowledge, was hitherto not described.

Case Report

A 70-year-old housewife in whom Gaucher' disease was diagnosed in 1940, was hospitalized in October 1975 due to weakness, arthralgia and purpura.

In 1961 she was splenectomized because of massive enlargement of the spleen with abdominal discomfort and marked thrombocytopenia. In 1962 she underwent left hemicolectomy because of adenocarcinoma of the transverse colon. According to her family physician, 1 month prior to her present admission she started to complain of vague abdominal pains and purpura in both legs. An upper gastrointestinal

barium series did not reveal esophageal varices, gastric neoplasm or duodenal ulcer. On admission, the patient was pale there was no jaundice the liver was palpated 8 cm below the costal margin, firm and nontender the abdomen was soft and no masses were felt. Symmetric purpuric lesions were seen below both knees.

The hemoglobin was 10.8 g/dl. The white cell count was 12,000 /l with a normal differential count and the platelet count was 120,000/ μ l. Serum iron was 62 μ g/dl transaminase (SGOT) 63 U (normal, approx. 40 U), LDH 267 U (normal, approx. 220 U) and alkaline phosphatase 98 U (normal approx. 80 U). Urea, glucose, electrolytes, albumin, globulin and protein immunoelectrophoresis were all in the normal range. The rheumatoid factor was +4 and Rose Waaler test was positive. 1640 LE cells were not found. A cryoprecipitate constituting more than 10% of the plasma volume was found and isolated. Its examination showed the presence of a mixed cryoglobulin consisting of a monoclonal IgM with κ light chains and a polyclonal IgG component. C_3 , C_4 and C_{1q} components of the complement were not detected in the cryoprecipitate. The serum C_3 , C_4 concentrations were 36 and 6 mg/dl respectively (normal C_3 90-200 mg/dl, C_4 30-60 mg/dl). The examinations of the patient's serum as well as the cryoprecipitate for Australian antigen and anti body were negative.

The mild anemia, the weight loss and the slight abdominal pain raised the suspicion of a recurrence of the colonic neoplasm. The rectoscopic examination (27 cm) was normal. A barium enema was performed and there was no evidence of malignancy; however 2 h later the patient complained of excruciating abdominal pain. A huge and tender mass appeared in the right lower abdominal quadrant with signs of acute abdomen. On laparotomy a large hematoma was found in the right rectal sheath the liver was enlarged, pale, firm and mottled. Because of the bleeding tendency a wedge liver biopsy was considered too dangerous and was therefore not performed. The histologic examination of the rectus muscle was normal. The postoperative course was uneventful however numerous nonsymmetric fresh purpuric crops appeared in the upper extremities. The histologic examination of a punch biopsy from an involved region revealed capillary damage with minimal extravasation of peripheral blood formed elements. Studies by the immunofluorescent antibody technique of a parallel section was negative for IgG, IgA and IgM, complement and fibrinogen. The previously observed purpuric lesions in both legs changed in color and became brown.

A few days later the patient complained of paresthesia and numbness in the 5th finger of the left hand. Sensory loss over this region was found as well as an impairment of abduction and adduction of the 5th finger. The electromyography disclosed that the motor nerve conduction velocities were in the low range for the left ulnar nerve, and the stimulation of this nerve elicited low amplitude response especially near the elbow. There was also disturbed motor conduction in the left hypothenar muscle, as compared with a normal one in the right thenar muscle. The median nerve showed a normal response. These findings were compatible with neuropathy of the ulnar nerve. The patient was treated with chlorambucil 4 mg/day and within 14 days the neuropathy disappeared.

During the follow-up lasting already 18 months there was no change in the cryoglobulinic pattern although the patient remained asymptomatic.

Discussion

In the previously reported patients with cryoglobulinemic neuropathy [1, 3, 4, 6, 7, 9, 11, 16, 18, 19] certain common features were apparent, i.e. (1) Peripheral neuropathy developed after recurrent purpuric episodes which often exacerbated prior to the onset of the neuropathy (2) A limited number of nerves were involved, mostly in the extremities, where the cutaneous manifestations were most prominent. (3) The sensory level of the neuropathy follows quite closely with the distribution of the purpuric rash or the brownish pigmentation. (4) In some of the cases the neurologic symptoms and signs appeared to become exacerbated during the winter and improved in the summer. Such seasonal fluctuations have been considered to be a necessary diagnostic feature of the cryoglobulinemic neuropathy [16].

In the presently reported patient the above mentioned features were prominent. In addition no evidence of other causes for peripheral neuropathy such as diabetes, uremia, carcinoma or connective tissue disease was found in our patient. It seems, therefore, that the most likely cause for the neuropathy was the cryoglobulinemia.

In the majority of patients the course of cryoglobulinemic neuropathy is of a progressive nature [16] although a remitting and relapsing course was also observed [16]. In our patient the neuropathy disappeared after a short while, following administration of chlorambucil, and no relapse was observed during a follow up of almost 2 years. It is possible, therefore, that the chlorambucil exerted a beneficial effect.

Most of the clinical manifestations of cryoglobulinemia are attributed to the precipitation of cryoglobulins in the small blood vessels, as well as to arterial and venous occlusions [5, 12, 13, 16]. Lacking histologic evidence, one can only speculate as to the mechanism underlying the cryoglobulinemic neuropathy in this patient, such as ischemic or hemorrhagic changes within the nerves [10, 16], cellular infiltration in the latter [2] or an allergic mechanism responsible for nerve demyelination [7].

Presumably the rectal sheath hematoma in this patient appeared following a strong effort of the abdominal muscles, related to the barium enema. As known, this rare complication is more common among elderly women and especially among those with blood dyscrasias [8, 20].

Cryoglobulinemia may appear during the course of several diseases such as periarthritis nodosa: the rectal sheath muscle biopsy showed no evidence for this entity. Moreover in this subject with Gaucher's disease

barium series did not reveal esophageal varices, gastric neoplasm or duodenal ulcer. On admission, the patient was pale, there was no jaundice, the liver was palpated 8 cm below the costal margin firm and nontender the abdomen was soft and no masses were felt. Symmetric purpuric lesions were seen below both knees.

The hemoglobin was 10.8 g/dl. The white cell count was 12,000 / μ l with a normal differential count and the platelet count was 120,000/ μ l. Serum iron was 62 μ g/dl, transaminase (SGOT) 63 U (normal approx 40 U) LDH 267 U (normal, approx 220 U) and alkaline phosphatase 98 U (normal approx. 80 U). Urea, glucose, electrolytes, albumin, globulin and protein immunoelectrophoresis were all in the normal range. The rheumatoid factor was +4 and Rose Waaler test was positive 1:640. LE cells were not found. A cryoprecipitate constituting more than 10% of the plasma volume was found and isolated. Its examination showed the presence of a mixed cryoglobulin consisting of a monoclonal IgM with λ light chains and a polyclonal IgG component. C_3 , C_4 and C_{1q} components of the complement were not detected in the cryoprecipitate. The serum C_3 , C_4 concentrations were 36 and 6 mg/dl respectively (normal C_3 90-200 mg/dl, C_4 30-60 mg/dl). The examinations of the patient's serum as well as the cryoprecipitate for Australian antigen and antibody were negative.

The mild anemia, the weight loss and the slight abdominal pains raised the suspicion of a recurrence of the colonic neoplasm. The rectoscopy examination (77 cm) was normal. A barium enema was performed and there was no evidence of malignancy; however, a later the patient complained of excruciating abdominal pains. A huge and tender mass appeared in the right lower abdominal quadrant with signs of acute abdomen. On laparotomy a large hematoma was found in the right rectal sheath the liver was enlarged, pale, firm and mottled. Because of the bleeding tendency a wedge liver biopsy was considered too dangerous and was therefore not performed. The histologic examination of the rectus muscle was normal. The postoperative course was uneventful however numerous nonsymmetric fresh purpuric crops appeared in the upper extremities. The histologic examination of a punch biopsy from an involved region revealed capillary damage with minimal extravasation of peripheral blood formed elements. Studies by the immunofluorescent antibody technique of a parallel section was negative for IgG, IgA and IgM, complement and fibrinogen. The previously observed purpuric lesions in both legs changed in color and became brown.

A few days later the patient complained of paresthesia and numbness in the 5th finger of the left hand. Sensory loss over this region was found as well as an impairment of abduction and adduction of the 5th finger. The electromyography disclosed that the motor nerve conduction velocities were in the low range for the left ulnar nerve and the stimulation of this nerve elicited low amplitude response, especially near the elbow. There was also disturbed motor conduction in the left hypothenar muscle, as compared with a normal one in the right thenar muscle. The median nerve showed a normal response. These findings were compatible with neuropathy of the ulnar nerve. The patient was treated with chlorambucil 4 mg/day and within 14 days the neuropathy disappeared.

During the follow-up lasting already 18 months there was no change in the cryoglobulinic pattern, although the patient remained asymptomatic.

Discussion

In the previously reported patients with cryoglobulinemic neuropathy [1 3 4 6, 7 9 11 16, 18 19] certain common features were apparent, i.e., (1) Peripheral neuropathy developed after recurrent purpuric episodes which often exacerbated prior to the onset of the neuropathy (2) A limited number of nerves were involved, mostly in the extremities, where the cutaneous manifestations were most prominent. (3) The sensory level of the neuropathy follows quite closely with the distribution of the purpuric rash or the brownish pigmentation. (4) In some of the cases the neurologic symptoms and signs appeared to become exacerbated during the winter and improved in the summer. Such seasonal fluctuations have been considered to be a necessary diagnostic feature of the cryoglobulinemic neuropathy [16].

In the presently reported patient the above mentioned features were prominent. In addition, no evidence of other causes for peripheral neuropathy such as diabetes, uremia, carcinoma or connective tissue disease was found in our patient. It seems, therefore, that the most likely cause for the neuropathy was the cryoglobulinemia.

In the majority of patients the course of cryoglobulinemic neuropathy is of a progressive nature [16] although a remitting and relapsing course was also observed [16]. In our patient the neuropathy disappeared after a short while, following administration of chlorambucil, and no relapse was observed during a follow up of almost 2 years. It is possible, therefore that the chlorambucil exerted a beneficial effect.

Most of the clinical manifestations of cryoglobulinemia are attributed to the precipitation of cryoglobulins in the small blood vessels, as well as to arterial and venous occlusions [5 12, 13 16]. Lacking histologic evidence, one can only speculate as to the mechanism underlying the cryoglobulinemic neuropathy in this patient, such as ischemic or hemorrhagic changes within the nerves [10, 16] cellular infiltration in the latter [] or an allergic mechanism responsible for nerve demyelination [7].

Presumably the rectal sheath hematoma in this patient appeared following a strong effort of the abdominal muscles, related to the barium enema. As known this rare complication is more common among elderly women and especially among those with blood dyscrasias [8, 20].

Cryoglobulinemia may appear during the course of several diseases such as periarthritis nodosa. the rectal sheath muscle biopsy showed no evidence for this entity. Moreover in this subject with Gaucher's disease

there was no evidence for the presence of other diseases known to be accompanied by cryoglobulinemia [14]

The question whether the cryoglobulinemia and the cryoglobulinemic neuropathy in this patient are related to Gaucher's disease, or are a mere coincidence, remains open. Further similar observations may help to elucidate this problem.

References

1. ABRAHAMSKY O and SLAVIN S. Neurologic manifestations in patients with mixed cryoglobulinemia. *Neurology Minn* 24: 245-249 (1974).
2. BARRON A. D. ROWLAND L. P. and ZAKSBERMAN H. M. Neuropathy with malignant tumor metastases. *J nerv ment Dis* 13: 10-31 (1960).
3. BOUDIN G et BARBIZET E. Cryoglobulinémie et lympho-réticulo-sarcome: observation d'un cas avec purpura nécrotique, syndrome de Raynaud, parotidite et névrites multiples. *Presse méd* 67: 594-597 (1959).
4. BROUET J. C. CLAUVEL J. P. DAVON F., ALFON M. and SELIGMAN M. Biologic and clinical significance of cryoglobulins. A report of 86 cases. *Am. J. Med* 57: 775-778 (1974).
5. CAPERTON E. M. and WILLIAMS R. C. *In vitro* study of IgM rheumatoid factor from mixed cryoglobulins. *J Lab Clin Med* 74: 239-249 (1969).
6. CREAM J. S. HERN J. E. C. HUOHLER R. A. C. and MACKENZIE I. C. H. Mixed or immune complex cryoglobulinemia and neuropathy. *J Neurol Neurosurg Psychiatr* 37: 82-87 (1974).
7. DAYAN A. D. and LEWIS P. D. Demyelinating neuropathy in macroglobulinemia. *Neurology Minn* 16: 1141-1144 (1966).
8. FRASER A. M. and COX S. Haematoma of rectus abdominis from the use of an exercise wheel: a report of 3 cases. *Br J Surg* 61: 577-579 (1974).
9. GARCIN R. MACLARMET J. et RONDOT P. Cryoglobulinémie et névrite multiples des membres inférieurs. *Revue neurol* 97: 142-146 (1967).
10. GARCIN R. MACLARMET J. et RONDOT P. Névrites dysglobulinémiques. *Presse méd* 70: 111-114 (1961).
11. GARCIN R., RONDOT P. et GRUFFER C. Cryoglobulinémie et péricarite noueuse à manifestations multineuritiques. *Revue neurol* 103: 589-592 (1960).
12. GOLDE D. and EPSTEIN W. Mixed cryoglobulin and glomerulonephritis. *Ann Intern Med* 69: 1221-1227 (1968).
13. GOLDBERG L. S. and BARNET E. V. Essential cryoglobulinemia: Immunologic studies before and after penicillamine therapy. *Archs Intern Med* 125: 145-150 (1976).
14. GRAY H. M. and KOHLER P. E. Cryoimmunoglobulins. *Semin. Haematol* 10: 87-112 (1973).
15. JORI G. P. and BUONAMANO G. Chronic hepatitis and cirrhosis of the liver in cryoglobulinemia. *Gut* 13: 610-613 (1972).
16. LOGOTHETIS J. KENNEDY W. R., ELLINGTON A. and WILLIAMS R. C. Cryo-

- globulinemic neuropathy: Incidence and clinical characteristics. *Archs Neurol.* 19: 389-397 (1968).
- 17 POWERS, J. H. Essential cryoglobulinemia manifested by peripheral gangrene and massive purpura. *Am. J. clin. Path.* 47: 61-64 (1958)
- 18 RISTOV, S. C., GRIVER, P. F. AND C. N. and STODOLSON, L. Reversal of systemic manifestations of cryoglobulinemia. Treatment with methylprednisolone. *Archs Intern. Med.* 136: 467-471 (1976)
- 19 SAKOUELE, F. A propos d'un cas de névrite cryoglobulinémique (étude clinique et biologique). *Sem. Hôp. Paris* 40: 33-36 (1964).
- 20 TITORE, C., LEPICUS, M., and KRAKAUER, J. S. 'Spontaneous' hematoma of the rectus abdominis muscle. Critical review of 50 cases with emphasis on early diagnosis and treatment. *Surgery* 72: 568-572 (1972)
- 21 VOLPE, R., ROBERTSON, A. B. FLITCHER, A. A., and CHARLES, W. B. Essential cryoglobulinemia: Review of the literature and report of a case treated with ACTH and cortisone. *Am. J. Med.* 20: 533-553 (1956).

Megakaryocytic Leukaemia

A Case of Unusual Outcome

W DROSZCZ, B MDZEWSKI, J WRONSKA, H POREMBINSKA
R JEDRZEJEWSKI and H BOGACKA ZATORSKA

Department of Pneumology Institute of Internal Diseases,
Department of Laboratory Diagnostics, Postgraduate Medical Education Centre and
Department of Angiosurgery and Transplantology Institute of Surgery
Department of Pathology Institute of Biostructure Medical Academy Warsaw

Key Words: Megakaryocytic leukaemia Megakaryocytes

Abstract In a 33-year-old woman with thrombocytosis, megakaryocytic hyperplasia in bone marrow, presence of megakaryocytes in peripheral blood and megakaryocyte-containing infiltrations in the liver, excellent result of Busulphan treatment (1 year full remission after Busulphan withdrawal) is reported. The authors suggest the diagnosis of megakaryocytic leukaemia.

Essential thrombocythaemia, primary thrombocythaemia, primary haemorrhagic thrombocythaemia, hyperthrombotic myelosis, and megakaryocytic leukaemia are synonyms of the syndrome described by Di Guglielmo in 1920 [1, 2].

Separation of megakaryocytic leukaemia from other myeloproliferative syndromes into which it has been included by DAMESHEK [3] is still a question at issue since frequently thrombocytosis is associated with certain phases of chronic myeloid leukaemia, osteomyelofibrosis or polycythemia vera being, however, only a transient sign in these diseases [4].

Case Report

A 33-year-old woman was admitted to the Institute of Surgery with signs of intestinal occlusion in March 26, 1976. On laparotomy extensive adhesions and an



Fig 1 Section of the liver showing 3 megakaryocytes HE \times 400

enormous liver were disclosed. Intraoperative histological examination revealed postnecrotic cirrhosis and presence of giant cells resembling morphologically megakaryocytes (fig 1).

Her haematological data were as follows: haemoglobin 146 g/dl, erythrocytes 4.6×10^6 l, haematocrit 45%, leucocytes 29×10^9 l with normal differential count, platelets 1.4×10^9 l. The patient was transferred to the medical department. Bone marrow examination disclosed significantly raised megakaryocyte count. Megakaryocytes were found in the peripheral blood (fig 2).

Philadelphia chromosome in peripheral bone blood cells was absent. Other laboratory data were typical of liver cirrhosis. Busulphan treatment (6 mg daily) was started on April 30, 1976. The course of treatment is illustrated in figure 3.

During the first day of M₂ the patient had slight left-sided hemiparesis (the platelet count was then 1×10^9 l). The dose of Busulphan was increased to 12 mg daily and hemiparesis slowly regressed.

At the end of May the clinical condition of the patient was critical. Episodes of disturbances of consciousness and hypotension which required intravenous noradrenaline drip occurred. After 1 week of such situation the patient's general condition started to improve. The patient was discharged on Busulphan, 12 mg/day in good condition on June 16, 1976 with platelet count of 100×10^9 l.

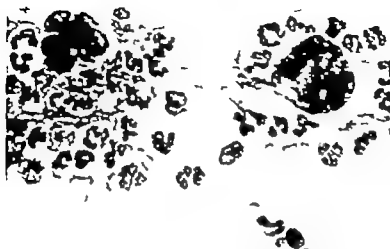


Fig Leucoconcentration of peripheral blood with 2 megakaryocytes.

During the subsequent observation Busulphan was given irregularly and completely withdrawn in February 1977. Since then the platelet counts varied between 10×10^9 and $15 \times 10^9/l$. No megakaryocytes were found in leucoconcentrates from peripheral blood and the bone marrow examination was normal.

At the time of submitting the paper for publication the general condition of the patient is good (she requires diuretics because of the cirrhosis of the liver), she is working in a household and cares for two children.

Discussion

Thrombocytosis associated with megakaryocyte hyperplasia in bone marrow and features of proliferation extramedullary megakaryocyte metaplasia in the liver, splenomegaly, presence of megakaryocytes in peripheral blood, high alkaline phosphatase activity in granulocytes and clinical observation lasting over 1 year without evidence of evolution into another myeloproliferative syndrome seem to be an adequate basis for the diagnosis of megakaryocytic leukaemia.

There was good response to cytostatic treatment with full haematological remission for more than 1.5 years continuing for almost 1 year after

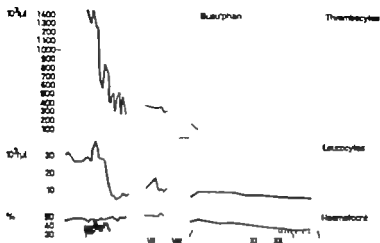


Fig 3 Platelet count, leucocyte count and haematocrit in the course of treatment with Busulphan

drug withdrawal. Clinical response included regression from hemiparesis and diminution of liver size and ascites.

References

- 1 Di Guglielmo, G. Eritroleucemia: puerile leukaemia. *Folia med. Napoli* 1: 36 (1920).
- 2 Ozer, P., Truax, W., Mirsch, D. and Lewis, W. Primary hemorrhagic thrombocythemia. *Am J Med* 28: 706 (1960).
- 3 Dameshek, W. Some speculation on the myeloproliferative syndromes. *Blood* 6: 372 (1951).
- 4 Hartwich, G., Schirmbeck, H. and Mödl, M. Thrombocythämie gibt es eine primäre Form. *Medische Klin* 70: 2005 (1975).

Book Reviews · Buchbesprechungen · Livres nouveaux

L. MOHR und R. STAEBLIN: *Handbuch der Inneren Medizin*, Band 2, Teil 3: Leukozytöses und retikuläres System I. Springer, Berlin 1976. XI + 303 pp., 124 fig., 50 tab. DM 340.- ISBN 3-540-07748-0.

This book is a volume of the series *Handbuch der Inneren Medizin*. It contains extensive reviews dealing with morphology, physiology and pathophysiology of the lymphocyte, the granulocyte, the monocyte/macrophage and the reticulo-endothelial system. Special emphasis is placed on light and electron microscopic findings, cytogenetics and kinetics. One chapter is devoted to technical aspects of cell counting. Each chapter represents a review of the many important contributions of the authors in their field and a summary of the work of other investigators. The literature is covered up to 1974. Abundant references are given for each chapter. The quality of the paper and of the photographs, some of which are in colour, is excellent. This book certainly represents the most extensive and careful compilation in this area in the German language. Clinical aspects will be covered in a forthcoming volume.

Due to the high price of the book and the large volume this publication will primarily be of interest to libraries and specialists in the field.

K. A. DRUGLIESS, Bern

R. PAOLETTI and S. SHERRY (eds.): *Thrombosis and Urokinase*. Academic Press, London 1976. VII + 257 pp. £ 8.20 ISBN 0-12-344960-7

This book represents the proceedings of a symposium held in Rome on October 30th to November 1st, 1975. It is constituted by 4 parts. The first is reserved to considerations on the physiology of fibrinolysis, the second dedicated to the chemistry and biochemistry of urokinase, the third partly to the activation of plasminogen by urokinase and other urokinase properties.

The fourth and main chapter is concerned with the therapeutic use of urokinase. Even though in 1975 the urokinase was available since only 10 years. The information collected on its therapeutic use covers a wide range of clinical applications. The different communications report with some detail the results obtained in pulmonary embolism, myocardial infarction, cerebrovascular disease, arterial and venous thrombosis and retinal vascular disease. These proceedings give in a condensed form a good review of the possibilities and limitations of urokinase as a therapeutic thrombolytic agent.

F. DUCKERT, Basel

A. STACHER und P. HÖCKER: *Erkrankungen der Myelopoese*. Urban & Schwarzenberg, München 1976. XVI + 360 pp., 275 fig., 186 tab. DM 180.- ISBN 3-541-07321-7

Dieses Buch fasst die Beiträge einer internationalen Arbeitstagung zur Leukämieforschung zusammen.

Der Schwerpunkt liegt beim myeloproliferativen Syndrom. Es wird versucht, die unter diesem Begriff zusammengefassten Krankheitsentitäten pathophysiologisch und pathologisch neu zu charakterisieren. Neue zytologische, zytogenetische, klinische

und biochemische Untersuchungen werden angelegt. Neue therapeutische Möglichkeiten werden anhand von klinischen und experimentellen Studien erörtert. Ebenfalls besprochen werden die akuten Leukämien, der derzeitige Stand der Chemotherapie, Immunotherapie und der unterstützenden Behandlung.

Sehr gut ist das Kapitel über Präleukämien im Kindes- und Erwachsenenalter.

Das Buch ist für hämatologisch und hämato-onkologisch tätige Ärzte als Nachschlagewerk zu empfehlen.

|| Sprick, Basel

A. H. GOLDSTONE. *Examination Haematology*. Saunders, Eastbourne 1977. VI + 206 pp., £ 4.95. ISBN 0-7216-4148-2.

In diesem Buch werden den Kandidaten für das angestrebte schriftliche Äquivalent des Facharztes Grundlagen der Hämatologie vermittelt. Es ist didaktisch sehr glücklich in 5 Teile gegliedert.

Im 1. Teil wird mit 50 Multiple-Choice-Fragen das allgemeine hämatologische Wissen getestet. Im 2. Teil wird anhand von über 50 typischen Blutbildern bzw. serologischen Befunden eine entsprechende Interpretation und Diagnose gefordert. Im 3. Teil ist diese Diagnose aufgrund von 10 Mikrophotographien von Blutausstrichen zu erstellen. Im 4. Teil folgt eine kurze Einführung über die wichtigsten Gerinnungsuntersuchungen, und daran anschließend folgen 20 Interpretationsfragen aufgrund klinischer Angaben. Im 5. Teil werden schließlich klinische Fragen der praktischen Hämatologie präzise und übersichtlich besprochen. Im ganzen handelt es sich um ein hervorragendes didaktisches Kompendium zur hämatologischen Fortbildung.

H. BRADWETTER, Innsbruck

J. NEJEDLIK and P. POČKA. *Regulation of Haemoglobin Synthesis*. Martinus Nijhoff, Den Haag, 1977. 206 pp. ISBN 90-247 1999-2.

The haematologists know the personal contribution of the authors to the problem of haem synthesis. This book is a translation from a document previously published in Czechoslovakia. The first three parts, devoted to the physiology and biochemistry of haemoglobin synthesis (iron uptake, haem synthesis and globin synthesis) are the most important. 130 of the 200 pages of this book. Perhaps, one could regret that the other sections are short: regulation of haemoglobin synthesis, differentiation of erythroid cells and anaemia due to disorders of haemoglobinization.

It is the opinion of the reviewer that for the three physiologic chapters on the mechanisms of haemoglobin synthesis, this book deserves its place in every library devoted to haematology or physiology.

Y. NARLIK, Paris

H. F. BURN, B. G. FORBES and H. M. RANBY. *Hemoglobinopathies. Major Problems in Internal Medicine*, vol. XII. Saunders, Philadelphia 1977. 308 pp. ISBN 0-7216-2179-1.

Another excellent review on hemoglobinopathies.

It is an important problem in Internal Medicine, all countries, because of the large population migrations, so that not only the specialists in haematology but also haematologists could read such a book.

All the chapters are clear well documented, and enriched with tables, schemes, and figures: structure and function of human hemoglobin, thalassemias, hemoglobin variants, sickle-cell anemia. Particularly interesting are the large chapters devoted to new aspects: instable hemoglobins, hemoglobinopathies with abnormal oxygen binding and M hemoglobins.

A large number of references (40 pages) will be a useful tool for the reader

J. NAJEAN, Paris

J. ALEXANDROWICZ and J. LISIEWICZ, *Hematology of Infectious Diseases*, Polish Medical Publishers, Warsaw 1976. XII + 359 pp. US\$ 9.00.

ALEXANDROWICZ and LISIEWICZ have managed to well summarize what the hematologist should know about blood changes in infectious diseases. Today's hematologists should be balanced between blood disorders *per se* as well as blood changes in nonhematological conditions. This booklet fulfills a real need in covering the second part of the hematologist's field of action. I can therefore fully recommend this book.

P. A. MITSCHER, Geneva

Varia

24. Jahrestagung der Deutschen Gesellschaft für Hämatologie und Onkologie gemeinsam mit der Österreichischen Gesellschaft für Hämatologie und Onkologie

Göttingen, 8–11 Oktober 1978

Hauptthemen. Der Erythrozyt Morphologie und Rheologie Therapie der Leukosen und malignen Lymphome, Funktionsstörungen der Granulozyten Hyperkoagulabilität, Knochentumoren – Definition und Therapie.

Anmeldung von Kurzvorträgen bis 31. Mai 1978 an: Prof. W. SCHÄDTER, Direktor der Universitäts-Kinderklinik, Humboldtallee 38, D-3400 Göttingen (BRD)

Acta haemat. 60: 129–136 (1978)

Differentiation Between Benign and Malignant Monoclonal Gammopathies by Discriminant Analysis on Serum and Bone Marrow Parameters¹

A. MORELL, W. MAURER, F. SEVAREL and S. BARANDUN

Institute for Clinical and Experimental Cancer Research of the University of Berne
and Wirtschafts-Mathematik AG, Zurich

Key Words. Monoclonal gammopathies · Discriminant analysis · Multiple myeloma · Waldenström's macroglobulinaemia

Abstract Bone marrow samples of 28 individuals with clinically benign and of 41 patients with malignant monoclonal gammopathy were analyzed for the total number of lymphoplasmocellular elements containing cytoplasmic immunoglobulins and for the monoclonal fraction of these cells. Monoclonal immunoglobulin components were determined in sera. A discriminant analysis was performed on the data: the variables were transformed and in a stepwise procedure used for the construction of a discriminant function which by dividing point allowed good distinction between the two groups of patients. By use of this discriminant function, 91% of the patients in the sample were correctly classified.

Differentiation between individuals with so-called benign monoclonal gammopathy (MG) and patients with malignant MG such as multiple myeloma or Waldenström's macroglobulinaemia is of crucial importance because of serious therapeutic consequences. The recognition of advanced malignant disease is possible by means of well-established criteria [6, 16, 18]. The diagnosis of early stages of malignant MG, however, is difficult. It often takes months or even years of observation and repeated X-ray and laboratory examinations to reliably discriminate between benign and malignant MGs. We report a new approach to this problem. It is based on a

This work was supported by grants of the Swiss National Science Foundation and the Angelo Bonolis-Fond.

Received: December 15, 1977; accepted: January 24, 1978.

All the chapters are clear well documented, and enriched with tables, schemes, and figures: structure and function of human hemoglobin, thalassemias, hemoglobin variants, sickle-cell anemia. Particularly interesting are the large chapters devoted to new aspects. Instable hemoglobins, hemoglobinopathies with abnormal oxygen binding and M hemoglobins.

A large number of references (40 pages) will be a useful tool for the reader

J. NAJMAN, Paris

J. ALEKSANDROWICZ and J. LISIEWICZ. Hematology of Infectious Diseases. Polish Medical Publishers, Warsaw 1976. Xii + 259 pp. US\$ 9.00

ALEKSANDROWICZ and LISIEWICZ have managed to well summarize what the hematologist should know about blood changes in infectious diseases. Today's hematologists should be balanced between blood disorders *per se* as well as blood changes in nonhematological conditions. This booklet fulfills a real need in covering the second part of the hematologist's field of action. I can therefore fully recommend this book.

P. A. MICHAEL, Geneva

Varia

24. Jahrestagung der Deutschen Gesellschaft für Hämatologie und Onkologie gemeinsam mit der Österreichischen Gesellschaft für Hämatologie und Onkologie

Göttingen, 8-11. Oktober 1978

Hauptthemen. Der Erythrozyt Morphologie und Rheologie Therapie der Leukosen und malignen Lymphome Funktionsstörungen der Granulozyten Hyperkoagulabilität Knochentumoren - Definition und Therapie.

Anmeldung von Kurzvorträgen bis 31. Mai 1978 an Prof. W. SCHAÖTTER, Direktor der Universitäts-Kinderklinik, Humboldtallee 38, D-3400 Göttingen (BRD).

SPSS package [4]. This program is based on a model assuming identical variance-covariance matrices in the two groups. Since the parameters S1, S3 and S4 have higher mean values and larger variances in the malignant group than in the benign group, logarithmic transformation was performed in order to achieve homoscedasticity. The relative clone size (S2) scatters widely in the benign group, ranging from 70 to 99% (almost uniformly distributed). In the malignant group 33 (72%) of the 46 patients have a value of $\geq 99\%$, 6 patients (13%) a value of 98%, and the rest (15%) values down to 87%. The most appropriate transformation for the parameter S2 was dichotomization into S2D, with S2D = 1 for S2 98% or more than 98%, and S2D = 0 for S2 less than 98%. The dividing point 98% gave the best separation between benign and malignant cases. The stepwise method of discriminant analysis was performed on the four transformed variables which were included into the discriminant function according to their partial multivariate F ratios. The dividing point was defined as the arithmetic mean between the centroids of the values for the two groups of patients obtained by the linear discriminant function.

Results

The two groups of patients differed significantly ($p < 0.001$) when the arithmetic mean values for lymphoplasmocellular BM elements (S1), for total serum protein (S3) and for the relative size of the serum M-gradient were compared (table I). Mean values for all three parameters are higher in the malignant group. Overlapping of individual values of the two groups, however, is evident.

Direct immunofluorescence analyses with Ig class- and light chain type-specific reagents allowed the identification of the cytoplasmic Ig in

Table I Comparison of serum and BM parameters between patients with benign and patients with malignant MG

	S1 % of nucleated BM cells	S3, g/dl	S4 % of total serum protein
Malignant MG (41 cases)			
mean \pm 1 SD	47 \pm 28	8.7 \pm 1.9	38 \pm 14
Range	2 - 98	4.5 - 13.3	11 - 70
Benign MG (28 cases)			
Mean \pm 1 SD	11 \pm 8	7.1 \pm 0.7	17 \pm 6
Range	1 - 32	5.1 - 8.0	9 - 33
Normal values	< 0.1 -	6.3 - 8.4	

discriminant analysis on serum and bone marrow (BM) parameters which were determined in individuals with benign and in patients with malignant MG

Patients and Methods

Patients

Serum and BM samples of 74 patients with MG were available. Their ages were between 30 and 83 years, both sexes were about equally represented. Differentiation between benign and malignant MG was made by the generally used diagnostic criteria [6, 16-18]

Patients with Malignant MG

Multiple myeloma was found in 34 patients. The M-component consisted of IgG in 19 and IgA in 10 patients. 3 patients suffered from Bence-Jones myeloma without detectable serum M-gradient but with high concentrations of urinary Bence-Jones protein. In 12 patients with monoclonal serum IgM, Waldenström's macroglobulinemia was diagnosed

Individuals with Benign MG

This diagnosis was established in 28 probands who had no significant clinical disturbances, no rise of the serum M-gradient and no increase of the BM infiltration with plasma cells or lymphoid elements during an observation period of at least 3 years. On average, BM infiltration and serum M-gradients were clearly less pronounced than in the group with malignant gammopathy (fig 1 table I). The M-gradient was IgG in 16 patients, IgA in 9 and IgM in 3 patients.

Immunochemical Analyses of Serum and Urine

The immunochemical tests performed on serum and urine samples are described in the previous paper [8].

Immunofluorescence Analyses of BM

Immunofluorescence analyses have been described in the previous paper [8]. The monoclonal cell population, i.e. plasma cells and lymphoid elements containing cytoplasmic immunoglobulins (Ig) could easily be identified in all samples. It was expressed in percent of all Ig-containing cells and termed the relative clone size.

Discriminant Analysis

The following serum and BM parameters were evaluated. S1 Plasma cells and lymphoid cells with cytoplasmic Ig in percent of nucleated BM cells. S2. Relative clone size: monoclonal cells in percent of all BM plasma cells or lymphoid cells containing cytoplasmic Ig. S3 Total serum protein in g/dl. S4 M-component in percent of the total serum protein.

The distributions of these four parameters differed statistically significantly between the two groups of patients. These differences were exploited for the construction of a discriminant function using the corresponding computer program from the

SPSS package [4]. This program is based on a model assuming identical variance-covariance matrices in the two groups. Since the parameters S1, S3 and S4 have higher mean values and larger variances in the malignant group than in the benign group, logarithmic transformation was performed in order to achieve homoscedasticity. The relative clone size (S2) scatters widely in the benign group, ranging from 70 to 99% (almost uniformly distributed). In the malignant group 33 (72%) of the 46 patients have value of $\geq 99\%$, 6 patients (13%) value of 98%, and the rest (15%) values down to 87%. The most appropriate transformation for the parameter S2 was a dichotomization into S2D with S2D = 1 for S2 98% or more than 98%, and S2D = 0 for S2 less than 98%. The dividing point 98% gave the best separation between benign and malignant cases. The stepwise method of discriminant analysis was performed on the four transformed variables which were included into the discriminant function according to their partial multivariate F ratios. The dividing point was defined as the arithmetic mean between the centroids of the values for the two groups of patients obtained by the linear discriminant function.

Results

The two groups of patients differed significantly ($p < 0.001$) when the arithmetic mean values for lymphoplasmocellular BM elements (S1), for total serum protein (S3) and for the relative size of the serum M-gradient were compared (table 1). Mean values for all three parameters are higher in the malignant group. Overlapping of individual values of the two groups, however is evident.

Direct immunofluorescence analyses with Ig class- and light chain type-specific reagents allowed the identification of the cytoplasmic Ig in

Table 1. Comparison of serum and BM parameters between patients with benign and patients with malignant MG

	S1, % of nucleated BM cells	S3, g/dl	S4 % of total serum protein
Malignant MG (41 cases)			
mean \pm 1 SD	47 \pm 28	8.7 \pm 1.9	38 \pm 14
Range	2 - 98	4.5 - 13.3	11 - 70
Benign MG (28 cases)			
Mean \pm 1 SD	11 \pm 8	7.1 \pm 0.7	17 \pm 6
Range	1 - 32	5.2 - 8.0	9 - 31
Normal values	< 0.1 - 2	6.5 - 8.4	

discriminant analysis on serum and bone marrow (BM) parameters which were determined in individuals with benign and in patients with malignant MG

Patients and Methods

Patients

Serum and BM samples of 74 patients with MG were available. Their ages were between 30 and 83 years, both sexes were about equally represented. Differentiation between benign and malignant MG was made by the generally used diagnostic criteria [6, 16-18]

Patients with Malignant MG

Multiple myeloma was found in 34 patients. The M-component consisted of IgG in 19 and IgA in 10 patients. 5 patients suffered from Bence Jones myeloma without detectable serum M gradient but with high concentrations of urinary Bence-Jones protein. In 12 patients with monoclonal serum IgM, Waldenström's macroglobulinemia was diagnosed

Individuals with Benign MG

This diagnosis was established in 28 probands who had no significant clinical disturbances, no rise of the serum M gradient and no increase of the BM infiltration with plasma cells or lymphoid elements during an observation period of at least 3 years. On average, BM infiltration and serum M-gradients were clearly less pronounced than in the group with malignant gammopathy (fig. 1 table I). The M-gradient was IgG in 16 patients, IgA in 9 and IgM in 3 patients.

Immunochemical Analyses of Serum and Urine

The immunochemical tests performed on serum and urine samples are described in the previous paper [8]

Immunofluorescence Analyses of BM

Immunofluorescence analyses have been described in the previous paper [8]. The monoclonal cell population, i.e. plasma cells and lymphoid elements containing cytoplasmic immunoglobulins (Ig), could easily be identified in all samples. It was expressed in percent of all Ig-containing cells and termed the relative clone size.

Discriminant Analysis

The following serum and BM parameters were evaluated: S1 Plasma cells and lymphoid cells with cytoplasmic Ig in percent of nucleated BM cells. S2 Relative clone size monoclonal cells in percent of all BM plasma cells or lymphoid cells containing cytoplasmic Ig. S3 Total serum protein in g/dl. S4 M-component in percent of the total serum protein.

The distributions of these four parameters differed statistically significantly between the two groups of patients. These differences were exploited for the construction of a discriminant function using the corresponding computer program from the

SPSS package [4]. This program is based on a model assuming identical variance-covariance matrices in the two groups. Since the parameters S1, S3 and S4 have higher mean values and larger variances in the malignant group than in the benign group, logarithmic transformation was performed in order to achieve homoscedasticity. The relative clone size (S2) scatters widely in the benign group, ranging from 70 to 99% (almost uniformly distributed). In the malignant group 33 (72%) of the 46 patients have values of $\geq 99\%$, 6 patients (13%) a value of 98%, and the rest (15%) values down to 87%. The most appropriate transformation for the parameter S2 was dichotomization into S2D, with S2D = 1 for S2 98% or more than 98%, and S2D = 0 for S2 less than 98%. The dividing point 98% gave the best separation between benign and malignant cases. The stepwise method of discriminant analysis was performed on the four transformed variables which were included into the discriminant function according to their partial multivariate *F* ratios. The dividing point was defined as the arithmetic mean between the centroids of the values for the two groups of patients obtained by the linear discriminant function.

Results

The two groups of patients differed significantly ($p < 0.001$) when the arithmetic mean values for lymphoplasmocellular BM elements (S1), for total serum protein (S3) and for the relative size of the serum M-gradient were compared (table I). Mean values for all three parameters are higher in the malignant group. Overlapping of individual values of the two groups, however, is evident.

Direct immunofluorescence analyses with Ig class- and light chain type-specific reagents allowed the identification of the cytoplasmic Ig in

Table I. Comparison of serum and BM parameters between patients with benign and patients with malignant MG

	S1 % of nucleated BM cells	S3 g/dl	S4 % of total serum protein
Malignant MG (41 cases)			
mean \pm 1 SD	47 \pm 28	8.7 \pm 1.9	38 \pm 14
Range	2 - 98	4.5 - 13.3	11 - 70
Benign MG (28 cases)			
Mean \pm 1 SD	11 \pm 8	7.1 \pm 0.7	17 \pm 6
Range	1 - 31	5.2 - 8.0	9 - 33
Normal values	< 0.1 - 2	6.5 - 8.4	

discriminant analysis on serum and bone marrow (BM) parameters which were determined in individuals with benign and in patients with malignant MG

Patients and Methods

Patients

Serum and BM samples of 74 patients with MG were available. Their ages were between 30 and 83 years, both sexes were about equally represented. Differentiation between benign and malignant MG was made by the generally used diagnostic criteria [6, 16-18]

Patients with Malignant MG

Multiple myeloma was found in 34 patients. The M-component consisted of IgG in 19 and IgA in 10 patients. 5 patients suffered from Bence-Jones myeloma without detectable serum M-gradient but with high concentrations of urinary Bence-Jones protein. In 12 patients with monoclonal serum IgM, Waldenström's macroglobulinemia was diagnosed.

Individuals with Benign MG

This diagnosis was established in 28 probands who had no significant clinical disturbances, no rise of the serum M-gradient and no increase of the BM infiltration with plasma cells or lymphoid elements during an observation period of at least 3 years. On average, BM infiltration and serum M-gradients were clearly less pronounced than in the group with malignant gammopathy (fig. 1 table 1) The M-gradient was IgG in 16 patients, IgA in 9 and IgM in 3 patients.

Immunoclinical Analyses of Serum and Urine

The immunochemical tests performed on serum and urine samples are described in the previous paper [8].

Immunofluorescence Analyses of BM

Immunofluorescence analyses have been described in the previous paper [8]. The monoclonal cell population, i.e. plasma cells and lymphoid elements containing cytoplasmic immunoglobulins (Ig), could easily be identified in all samples. It was expressed in percent of all Ig-containing cells and termed the relative clone size

Discriminant Analysis

The following serum and BM parameters were evaluated. S1 Plasma cells and lymphoid cells with cytoplasmic Ig in percent of nucleated BM cells. S2. Relative clone size, monoclonal cells in percent of all BM plasma cells or lymphoid cells containing cytoplasmic Ig. S3 Total serum protein in g/dl. S4 M-component in percent of the total serum protein.

The distributions of these four parameters differed statistically significantly between the two groups of patients. These differences were exploited for the construction of a discriminant function using the corresponding computer program from the

Table II. Discrimination between benign and malignant MG on the basis of the relative clone size

Number of cases	Relative clone size monoclonal cells in % of all lymphoplasmocellular elements in the BM				Clinical evaluation
	> 99%	98%	97%	< 97%	
45	33	6	2	5	malignant
28	3	1	3	21	benign

Optimal discrimination if the dividing point is taken at 98%. Malignant MG if clone > 98%. Benign MG if clone < 98%.

parameters (table I) were included. This was done by a discriminant analysis on the transformed variables S1, S2, S3 and S4. Data of the 5 patients with Bence Jones myeloma were rejected because they had no measurable serum M-component. The first variable included into the function was S2D (F value 65.9). It was followed by \log_{10} S1 and by \log_{10} S4 (F values 17.1 and 7.1). Both variables brought additional information for the construction of the function. S3 however (F value 0.03), did not bring any further improvement of the discriminant power and was therefore disregarded.

The resulting discriminant function reads

$$D(S1, S2, S4) = 5.99 - 1.39 \log_{10} S1 - 1.37 S2D - 2.42 \log_{10} S4 \\ = 5.99 - 1.39 \log_{10} S1 - 2.42 \log_{10} S4 \begin{cases} 1.37 \text{ (if clone } > 98\%) \\ 0 \text{ (if clone } < 98\%). \end{cases}$$

The arithmetic mean values for $D(S1, S2, S4)$ were 1.59 in the benign group and -1.08 in the malignant group. The dividing point \bar{D} giving an optimal separation between malignant and benign cases was: $\bar{D} = 0.25$.

Therefore, if a value for $D(S1, S2, S4)$, calculated from the data of a given patient is below 0.25 the MG is likely to be malignant. If \bar{D} is higher than 0.25 the MG is probably benign. Figure 2 shows the values for $D(S1, S2, S4)$ calculated for the 69 individuals. Two distinct populations emerged, one for benign the other for malignant MG, each of them having an approximately symmetric distribution. 63 patients were correctly classified (91.3%) by use of this function. Thus, results were markedly better than when the discrimination was made on the basis of one parameter only: 3 patients with malignant and 3 individuals with clinical-

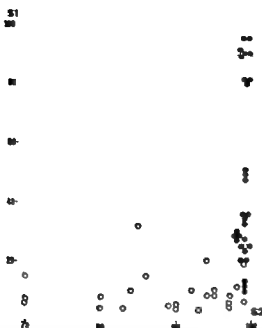


Fig 1 The relationship between the total number (S1) and the monoclonal fraction (S2) of lymphoplasmocellular elements in the BM. O = individuals with benign MG ● = patients with multiple myeloma or Waldenström's macroglobulinemia.

BM plasma cells and large lymphoid cells. Figures for the percentage distribution of IgG, IgA, IgM, and λ Ig-containing cells in normal BM have been reported [8]. In patients with MG the percentage distribution of these Ig-containing cells was found to be shifted by more than 3 SD in favor of the monoclonal lymphoplasmocellular elements. This monoclonal fraction of the Ig-containing BM cells, termed the relative clone size (S2) is compared to S1 (Ig-containing BM cells in percent of nucleated BM cells) in figure 1. With few exceptions, the relative clone size was higher in patients with malignant disease than in benign conditions. In most malignant cases, it ranged in the order of 99% of the Ig-containing cells, irrespective of the total number of BM plasma cells or lymphoid cells (S1). On the basis of this observation, parameter S2 was used for discrimination between the two groups. When the values were grouped as in table II, a relative clone size of 98% gave an optimal discrimination. With this dividing point of 98%, only 7 patients of the malignant group and 4 of the benign group were wrongly classified. Considerable improvement of the discrimination was achieved when the other serum and BM

Table II. Discrimination between benign and malignant MG on the basis of the relative clone size

Number of cases	Relative clone size: monoclonal cells in % of all lymphoplasmocellular elements in the BM				Clinical evaluation
	> 99%	98%	97%	< 97%	
46	33	6	2	5	malignant
28	3	1	3	21	benign

Optimal discrimination if the dividing point is taken at 98%. Malignant MG, if clone > 98%. Benign MG, if clone < 98%.

parameters (table I) were included. This was done by a discriminant analysis on the transformed variables S1, S2, S3 and S4. Data of the 5 patients with Bence Jones myeloma were rejected because they had no measurable serum M-component. The first variable included into the function was S2D (F value 65.9). It was followed by \log_{10} S1 and by \log_{10} S4 (F values 17.1 and 7.1). Both variables brought additional information for the construction of the function. S3 however (F value 0.03), did not bring any further improvement of the discriminant power and was therefore disregarded.

The resulting discriminant function reads:

$$D(S1, S2, S4) = 5.99 + 1.39 \log_{10} S1 - 1.37 S2D - 2.42 \log_{10} S4 \\ = 5.99 + 1.39 \log_{10} S1 - 2.42 \log_{10} S4 - \begin{cases} 1.37 & (\text{if clone} > 98\%) \\ 0 & (\text{if clone} < 98\%). \end{cases}$$

The arithmetic mean values for $D(S1, S2, S4)$ were 1.59 in the benign group and -1.08 in the malignant group. The dividing point S giving an optimal separation between malignant and benign cases was: $S = 0.25$.

Therefore, if a value for $D(S1, S2, S4)$, calculated from the data of a given patient is below 0.25 the MG is likely to be malignant. If it is higher than 0.25 the MG is probably benign. Figure 2 shows the values for $D(S1, S2, S4)$ calculated for the 69 individuals. Two distinct populations emerged, one for benign the other for malignant MG, each of them having an approximately symmetric distribution. 63 patients were correctly classified (91.3%) by use of this function. Thus, results were markedly better than when the discrimination was made on the basis of one parameter only: 3 patients with malignant and 3 individuals with clinical-

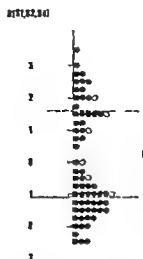


Fig 2 Distribution of the values for $D(S_1, S_2, S_4)$ calculated from the data of 69 patients with either benign or malignant MG --- = Centroids of the two groups; — = dividing point S ● = correctly classified patients; ○ = patients assigned to the wrong group

ly benign MG were assigned to the wrong group (fig. 2). Careful reexamination of clinical and laboratory data cast some doubt on the actual diagnosis in some of these 6 cases which will be clarified by follow-up studies.

Discussion

Our efforts are aimed at the early recognition of malignant MG. Its distinction from benign conditions is difficult [17-19] but highly desirable. Patients in early stages are known to respond better to chemotherapy than patients with widespread myelomatosis [13]. Cytostatic agents on the other hand have to be avoided in cases with benign MG. The two groups of MG are known to differ with respect to various seizable laboratory parameters directly or indirectly related to the expansion of the monoclonal cells. Such differences comprise serum concentration of the M-component, total serum protein and grade of BM infiltration with lymphoplasmocellular elements [6, 16-19]. They foster speculations that the two types of lymphoplasmocellular dyscrasias [11] may represent different pathophysiological entities, although transition from benign to malignant state is documented for a few cases [1, 2, 5, 9, 10]. Individual values

for each of these parameters show considerable overlapping between the two groups. The same holds for other parameters, related to the humoral immune deficiency states which are frequently observed in patients with malignant MG. Serum levels of polyclonal Ig [14, 18, 19] and numbers of polyclonal B-lymphocytes bearing normal surface Ig [3, 7, 12, 15] are generally lower in patients with malignant than in individuals with benign MG.

None of these variables *per se* is a suitable tool for an appropriate distinction between the two groups. If they are combined in a linear function, however, their discriminant power is amplified in such a way that a discrimination between benign and malignant cases is possible. The exact probability of a correct classification is not yet established. It has to be determined by a cross validation with new data which are currently being collected.

Acknowledgements

We wish to express our gratitude to Miss Monique Nef for her skilful technical assistance, and Dr. H. P. Röst, Wirtschaftsmathematik AG, Zürich, for helpful advice.

References

- 1 AXELSON, U. and HILLEM, J. A population study on monoclonal gammopathy. Follow-up after 5 / years on 64 subjects detected by electrophoresis on 6995 sera. *Acta med. scand.* 191: 111-113 (1972).
- 2 AZAR, H. Pathology of multiple myeloma and related growth, in AZAR and PORTER Multiple myeloma and related disorders, pp. 1-85 (Harper & Row New York 1973).
- 3 BRODER, S., HUMPHREY R., DUNN, M., BRACEMAN, M., MEADE, B., GOLDMAN, C., STROBER, W. and WALDMAN, T. Impaired synthesis of polyclonal (nonparaprotein) immunoglobulins by circulating lymphocytes from patients with multiple myeloma. *New Engl. J. Med.* 293: 887-892 (1975).
- 4 KLECKA, W. R. Discriminant analysis: in NIE, HULL, JENKINS, STEINBERGER and BENT Statistical packages for the social sciences; 2nd ed., chap. 23, pp. 434-457 (McGraw Hill, New York 1975).
- 5 KYLE, R. A. and BAYRD, E. D. Benign monoclonal gammopathy. A potentially malignant condition? *Am. J. Med.* 40: 436 (1966).
- 6 KYLE, R. A. and BAYRD, E. D. Multiple myeloma. Basic series and clinical features; in KYLE and BAYRD The monoclonal gammopathies. Multiple myeloma and related plasma-cell disorders, pp. 75-135 (Thomas, Springfield 1976).

- 7 LINDSTRÖM, P. D. HARDY W. R. EBERLE, B. J., and WILLIAMS, R. C. Multiple myeloma and benign monoclonal gammopathy. Differentiation by immunofluorescence of lymphocytes. *Ann. Intern. Med.* 78 837-844 (1973)
- 8 MORELL, A. SKVAREL, P. HUBER, H. J. SPENGLER, G. and BARANDUN, S. Cytoplasmic immunoglobulins in bone marrow cells of polyclonal and of monoclonal origin. *Acta haemat.* 60 137-147 (1978)
- 9 NORGGAARD, O. Recherches sur l'évolution préclinique du myélome multiple. *Acta med. scand.* 176. 137-141 (1964).
- 10 NORGGAARD, O. Three cases of multiple myeloma in which the preclinical asymptomatic phases persisted throughout 15 to 24 years. *Br J Cancer* 25 417-422 (1971).
- 11 OSTERMAN E. F., Multiple myeloma and related plasma cell dyscrasias; in SAMTER and *Ass. Immunological diseases* (Little, Brown, Boston 1971).
- 12 FREUD-HOMME, J. L. HUREZ, D., DANON, F. BROUET J. C., and SELIGMANN M. Intracytoplasmic and surface-bound immunoglobulins in 'non-secretory' and Bence Jones myeloma. *Clin. exp Immunol.* 25 428-436 (1976)
- 13 SALMON S. E. Immunoglobulin synthesis and tumor kinetics of multiple myeloma. *Semin. Hemat.* 10 135-147 (1973).
- 14 SALMON, S. E. and SELIGMANN, M. B-cell neoplasia in man. *Lancet* II. 1230-1233 (1974).
- 15 SELIGMANN, M., FREUD-HOMME, J. L., and BROUET J. C. B and T cell markers in human proliferative blood diseases and primary immunodeficiencies, with special reference to membrane bound immunoglobulins. *Transplants Rev* 16 85-113 (1973).
- 16 SHAPPER, L. and HAJN A. Myelomatosis, fundamentals and clinical features, pp. 10-25 49-100, 138-188 (Karger Basel 1971).
- 17 UICC Technical Report Series in WARNER, POTTER and METCALF Multiple myeloma and related immunoglobulin-producing neoplasms, pp. 20-26 (Geneva, 1974).
- 18 WALDENSTRÖM J., Diagnosis and treatment of multiple myeloma, pp 15-153 (Grune & Stratton, New York 1970).
- 19 ZAWADSKI, Z. A. and EDWARDS, G. A. Nonmyelomatous monoclonal immunoglobulinemia in SCHWARTZ *Progress in clinical immunology* vol 1 pp. 105-156 (Grune & Stratton, New York 1972)

Cytoplasmic Immunoglobulins in Bone Marrow Cells of Polyclonal and of Monoclonal Origin¹

A. MORELL, F. SEVARIL, H. J. HUSER, G. SPENGLER and S. BARANDUN

Institute for Clinical and Experimental Cancer Research, University of Berne, and
Institute for Clinical Protein Research, University of Berne, Tiefenau Hospital,
Berne

Key Words. Bone marrow plasma cells · Cytoplasmic immunoglobulins · Monoclonal plasma cells · Polyclonal plasma cells

Abstract. Cytoplasmic immunoglobulins in human bone marrow plasma cells and lymphoid cells were characterized by direct immunofluorescence with fluorochrome-labelled reagents specific for immunoglobulin heavy and light chains. The percentage distribution of cells containing IgA, IgG or IgM and κ - or λ -immunoglobulins was determined in bone marrow samples from 168 immunologically normal individuals, in 11 patients with polyclonal increase of bone marrow plasma cells and in 80 patients with benign or malignant monoclonal gammopathies. A clear differentiation between monoclonal and polyclonal cell populations could be obtained in all cases.

An increase of immunoglobulin (Ig) containing bone marrow (BM) cells may be due to monoclonal or to polyclonal proliferation. A monoclonal proliferation is compatible with the clinical diagnosis of benign or malignant gammopathy; a polyclonal increase is encountered in chronic inflammatory conditions. In most instances, these two types of cell expansion can readily be distinguished. homogeneous M components are found in serum and/or urine samples of patients with monoclonal proliferation [12]. Furthermore, a monoclonal proliferation is assumed when plasma cells exceed 10-15% of the nucleated BM cells, and particularly when sheets and clusters of immature and abnormal cells are seen [7, 16, 17].

This work was supported by grants of the Swiss National Science Foundation and the Angela Boscolasco-Fund².

Received: December 5, 1977; accepted: January 24, 1978.

There are however cases where such a distinction is difficult: a homogeneous Ig component may be rudimentary or absent in spite of monoclonal plasma cells, and thus be missed by conventional methods [6, 9]. Moreover BM plasmacytosis may be modest [7]. In these situations, the determination of cytoplasmic Ig in plasma cells and large lymphoid cells in the BM [4, 6] opens potentialities which have not yet been fully explored. It is the purpose of the present paper to fill this gap by a systematic analysis.

Material and Methods

Patients and BM Samples

BM aspirations were performed for diagnostic purposes in 168 patients hospitalized for various reasons. Since no proliferative disorders of lymphocytes or plasma cells nor abnormalities of serum Ig's could be found, these patients were considered as immunologically normal controls.

BM aspirations were analyzed in a second group comprising 11 patients who had elevated numbers of BM plasma cells. The clinical diagnosis is given in table II. No monoclonal Ig components were detectable in their serum or urine samples.

BM samples could be obtained from 80 patients with benign or malignant monoclonal gammopathies. Their age was between 30 and 83 years. Of these patients, 48 suffered from multiple myeloma or Waldenström's macroglobulinemia. In 32 patients, a benign monoclonal gammopathy was diagnosed. The generally used diagnostic criteria were applied [7, 16, 17]. Serum and urine analyses revealed the presence of M components in 74 of these patients. In 6 patients, no homogeneous Ig component was found and the monoclonal cells were assumed to be in a 'nonsecretory' state.

Preparation of BM Slides

The method for the preparation of BM slides has extensively been described [5, 8]. The following modifications were introduced. BM suspensions were adjusted to 2×10^6 nucleated cells/mL. From this suspension, identical monolayer glass slides containing approximately 10^6 nucleated cells were prepared in a Shandon-Elliott cytocentrifuge by application of 50 μ l cell suspension per sample holder.

Analysis of Serum and Urine Samples

M components in sera and in 50 or 100 times concentrated urines were identified by electrophoresis on cellulose acetate membranes and by immunoelectrophoresis with class and light chain type specific antisera. M components in sera were expressed in percent of the total serum protein. In sera from patients with rudimentary or no M components, concentrations of IgA, IgG and IgM were determined in radial immunodiffusion plates (Partigen plates).

Immunofluorescence Analyses

Reagents specific for IgA, IgG and IgM heavy chains and for κ and λ light chains were prepared, labelled with the fluorochromes FITC and/or TRITC and

tested for specificity on monoclonal BM plasma cells [5]. In addition, an antiserum reacting with all Ig heavy chains and both light chain types, termed anti-Ig, was labelled with FITC. Optimal dilutions of the conjugates, 1:20 to 1:40, were established in preliminary studies.

Fluorescence microscopy All preparations were examined under fluorescence microscope using incident illumination as described [8].

Evaluation of the BM slides. The total number of Ig-producing cells was determined by counting all cells with cytoplasmic staining for FITC-anti-Ig. Since slide contained approximately 10^5 nucleated cells, percentages for Ig-positive cells could be calculated. Figures obtained by this method generally agreed with plasma cell counts in May-Grimwald-Giemsa stained routine slides.

Calculation of the percentage of cells positive for particular heavy chain class or light chain type Different slides of an individual BM were stained with the respective conjugates. The sum of IgG, IgA and IgM and the sum of κ - and λ -positive cells were taken as 100%, and the percentage of cells with cytoplasmic Ig of particular heavy chain class or light chain type was calculated [4].

Results

Immunologically Normal Individuals

The arithmetic mean value and standard deviations for Ig-positive cells per BM slide in the control group of 168 individuals were 448 ± 290 . Since each slide contained approximately 10^5 nucleated cells, this figure means that roughly 0.4–0.5% of the nucleated BM cells contained cytoplasmic Ig. Morphologically these cells were predominantly plasma cells. Large lymphoid cells with a rim of fluorescent cytoplasm, however, were also observed and counted.

Percentage distribution of cells with cytoplasmic Ig of the various classes and types are given in table I. Since distributions were close to normal, arithmetic mean values and standard deviations could be calculated. IgD- and IgE-positive BM cells were neglected.

Patients with Increased Numbers of Ig-Producing BM Cells of Polyclonal Origin

Figures for the total number of Ig-containing BM cells of these patients and for the intracellular heavy and light chain distribution are presented in table II. Serum electrophoresis revealed unremarkable serum protein patterns in 9 patients. In 2 patients (Mos and Isc in table II), diffuse hypergammaglobulinemia was detected. Quantitative Ig determination showed a moderate elevation of IgA and/or IgG in the serum of some patients, whereas IgM was within normal limits. The cytoplasmic heavy

Table I Cytoplasmic Ig in BM cells from 163 immunologically normal individuals percentage distribution of IgA, IgG IgM κ and λ containing cells

Sum of IgA IgG IgM κ IgA IgG IgM Sum of κ and λ containing cells							
100		44 \pm 8	50 \pm 9	6 \pm 3	100		61 \pm 9 39 \pm 9

Table II Patients with increased numbers of Ig-containing BM cells of polyclonal origin

Patient	Cells with cytoplasmic Ig in % of nucleated BM cells	Percentage distribution of cells containing Ig heavy and light chains					Clinical diagnosis
		IgA	IgG	IgM	κ	λ	
Bae	4	50	46	4	57	43	lupus erythematosus
Mos	4	57	40	3	53	45	lupus erythematosus
Stu	5	49	46	5	68	3	bronchogenic carcinoma
Rot	6	56	43	1	66	34	solitary plasmocytoma ¹
Urf	8	41	58	1	71	29	lymphoproliferative disorder
Woe	9	42	55	3	44	56	tuberculosis of the lung
Sau	9	49	47	4	54	46	hypernephroma
Gra	10	32	67	1	53	45	primary amyloidosis
Ise	12	50	49	1	70	30	malignant lymphoma
Lin	12	56	41	3	61	36	chronic osteomyelitis
Bal	13	57	40	3	53	47	pancytopenia

¹ Solitary osteolytic lesion in the fifth cervical vertebra. Values reported in table II are from BM taken at the iliac crest.

and light chain distribution in the BM cells was clearly polyclonal in all 11 patients and percentage values were close to those observed in the control group (table I). In addition, the clinical diagnosis is given in table II.

Patients with Increased Numbers of Ig-Producing BM Cells of Monoclonal Origin

BM serum and urine samples from 74 patients with the clinical diagnosis of multiple myeloma, Waldenström's macroglobulinemia or benign monoclonal gammopathy were analyzed. The results of the immunochem

Table III. Immunohistochemical characterization of the M component in 74 patients with benign or malignant monoclonal gammopathy

M component		Number of cases
IgG	IgG/ κ	18
	IgG/ λ	15
IgA	IgA/ κ	6
	IgA/ λ	13
IgM	IgM/ κ	11
	IgM/ λ	4
Biclonal gammopathies	IgG/ κ + IgA/ κ	2
Bence-Jones myelomas	κ	4
	λ	1

ical characterization of the M components are given in table III. In most, but not in all patients, the number of BM cells with cytoplasmic Ig was significantly increased. In figure 1 the relative numbers of cells with cytoplasmic Ig are plotted against the relative size of the M gradient in the serum of the patients. A linear relationship can be seen. high M gradients are found in patients with high numbers of Ig-containing BM cells and small or rudimentary M gradients are found in cases with low counts of Ig-positive BM cells.

Cytoplasmic immunofluorescence with heavy and light chain specific reagents disclosed a clear-cut shift in the percentage distribution in favor of cells containing one of the three Ig heavy and one light chain. This deviation of more than 3 SDs from the normal percentage distribution (table I) was taken as the manifestation of monoclonality. Examples for monoclonal BM cells with either cytoplasmic IgA, IgG or IgM are listed in table IV. In all patients, the monoclonal cells could unequivocally be identified. The results completely agreed with the immunohistochemical analyses given in table III. In 62 of the 74 patients the monoclonal cells included 90% or more, in 36 patients 99% or more of all cytoplasmic Ig-containing cells. In all samples, however some plasma cells with other Ig than that synthesized by the monoclonal population were found, representing cells that produce the normal background Ig. Figure 2 shows the relationship between the monoclonal population (expressed in percent of all Ig-containing cells) and the total number of Ig-containing cells (expressed in percent of the nucleated BM cells). When plasma cells and/or lymphoid

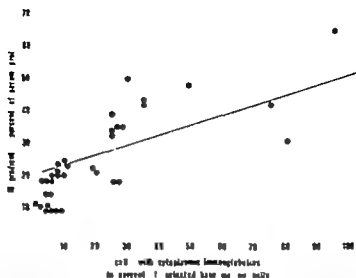


Fig 1 Scatter diagram showing the relationship between the relative size of the M gradient (in percent of serum protein) and the total number of cytoplasmic Ig-containing BM cells (in percent of the nucleated BM cells). Points represent serum and BM values of 69 patients with monoclonal BM cells. Patients with Bence-Jones myeloma and 'nonsecretory' myeloma are excluded. Regression line: $y = 0.31 + 19.79x$ correlation coefficient, $r = 0.58$.

Table IV BM with Ig-containing cells of monoclonal origin

Patient	Cells with cytoplasmic Ig in % of nucleated BM cells	Percentage distribution of cells containing Ig heavy and light chains				
		IgA	IgG	IgM	κ	λ
Stb	95	< 1	> 99	< 1	> 99	< 1
Fon	53	1	99	< 1	99	1
Tlc	26	6	94	< 1	97	3
Bur	3	6	93	1	10	90
Sch	90	> 99	< 1	< 1	< 1	> 99
Kun	48	99	1	< 1	1	99
Scn	77	96	4	< 1	1	99
Dle	5	90	9	1	95	5
Wao	90	1	1	98	> 99	< 1
Stc	30	< 1	< 1	> 99	> 99	< 1
Kln	30	3	5	92	99	1
Wcy	8	2	3	95	95	5

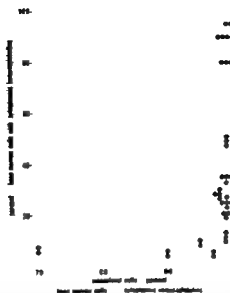


Fig 2. Relationship between total numbers of plasma cells (lymphoid cells) and monoclonal plasma cells (lymphoid cells) in 74 patients with benign or malignant monoclonal gammopathy. Ordinate, total number of cells containing cytoplasmic Ig (in percent of nucleated BM cells); abscissa, percentage of monoclonal Ig-containing cells.

Table V Patients with monoclonal 'nonsecretory' Ig-containing cells

Patient	Cells with cytoplasmic Ig in % of nucleated BM cells	Percentage distribution of cells containing Ig heavy and light chains					Clinical diagnosis
		IgA	IgG	IgM	λ	μ	
Cem	3	81	11	8	82	18	benign monoclonal 'nonsecretory' plasma cell leukemia
Arm	10	6	81	1	95	5	benign monoclonal 'nonsecretory' plasma cell leukemia
Sch	15	1	1	< 1	> 99	< 1	'nonsecretory' myeloma
Sta	20	1	< 1	< 1	> 99	< 1	'nonsecretory' myeloma
Hed	35	98		< 1	< 1	< 1	'nonsecretory' myeloma
Vba	90	< 1	< 1	< 1	> 99	< 1	nonsecretory myeloma

cells with cytoplasmic Ig were elevated to more than 35% of the nucleated BM cells, the monoclonal fraction contained 99% or more. In patients with a moderate increase of Ig-containing BM cells, the monoclonal fraction scattered between 70% and more than 99% of all Ig-positive cells.

Patients with monoclonal nonsecretory plasma cells: in 5 patients where careful serum and urine analyses showed no evidence for a paraprotein or a Bence-Jones protein, a monoclonal population of Ig-synthesizing BM cells could be demonstrated.

Discussion

Immunologically Normal Individuals

The mean value for Ig-containing BM cells ascertained by cytoplasmic immunofluorescence with FITC-anti Ig was approximately 0.45% of the nucleated cells. As expected from morphological correspondence, this figure agrees with normal values for plasma cells in routine BM smears stained by standard techniques [19]. The percentage distribution of BM cells with intracellular heavy and light chains shown in table I is similar to values reported earlier for BM [4-15] and comparable to values observed in spleen and tonsils [2, 3, 10-15]. It is known that molecules of the various Ig classes differ in their rate of catabolism and in the distribution within body compartments [18]. When these differences are considered, the percentage distribution of Ig-containing BM cells corresponds to the relative Ig serum concentrations [4].

Patients with Increased Numbers of Ig-Producing BM Cells of Polyclonal Origin

At the time when BM was obtained from the patients listed in table II the definitive diagnosis was not established and the possibility of an early stage of myeloma or related disorders and particularly of nonsecretory myeloma, had to be considered. The percentage distribution of the BM plasma cells with regard to Ig classes and types, however, was clearly polyclonal.

Patients with Increased Numbers of Ig-Producing BM Cells of Monoclonal Origin

Between the magnitude of the M gradient expressed in percent of serum protein and the total number of Ig cells, a linear relationship could be

established (fig. 1). Of special interest for the present study were patients with close-to-normal or moderately elevated numbers of Ig-containing BM cells and rudimentary serum M gradients. Even in such cases, the identification of the monoclonal population by cytoplasmic immunofluorescence posed no problems (fig. 2) implying that the monoclonal cells are widely dispersed throughout the BM. It has been our experience that in individuals with benign monoclonal gammopathy the relative clone size tended to be smaller i.e. between 70 and 95% of all Ig-synthesizing cells, than in patients with malignant gammopathy. In the majority of the latter group, 99% or more of the Ig-positive cells were monoclonal, irrespective of the total number of plasma cells or lymphoid cells and independent of the magnitude of the M component.

In about 1% of the patients with multiple myeloma, no paraprotein and no free urinary light chains are detectable [9]. In most of these 'nonsecretory' myelomas, the monoclonal plasma cells can be demonstrated by immunocytochemical methods [1, 6, 11, 13, 14]. In 2 of our 6 patients with monoclonal 'nonsecretory' BM cells (table V) no signs of clinical deterioration could be observed during 3 years and a benign monoclonal condition was thus assumed. The other 4 patients suffered from progressive multiple myeloma.

No cytoplasmic heavy chains were detectable in the monoclonal cells of patients Sch, Sta and Vba, and no light chains in the cells of patient Hel (table V). Cytoplasmic immunofluorescence staining was rather faint in the cells of Sch and Sta. Possibly this weak or even absent reactivity with the fluorochrome-conjugated reagents is due to a structural abnormality of the Ig chains. Such an abnormality could be related to the non-secreting state of the plasma cells.

In conclusion, some remarks concerning the diagnostic value of this technique have to be made: determinations of the percentage distribution of BM cells with regard to cytoplasmic heavy and light chains yielded relevant and reproducible results both in monoclonal and in polyclonal situations. In our hands, the method has proven to be useful in patients with moderately elevated numbers of BM cells where the possibility of monoclonal Ig-producing cell populations had to be considered and in patients who were suspected to have 'nonsecretory' myeloma. Furthermore it is suitable for following the clinical course in patients with benign monoclonal gammopathy. No additional information, however, is obtained in patients who suffer from advanced malignant disease with densely infiltrated BM, marked homogeneous Ig components and other clinical, X-ray

cells with cytoplasmic Ig were elevated to more than 35% of the nucleated BM cells, the monoclonal fraction contained 99% or more. In patients with a moderate increase of Ig-containing BM cells, the monoclonal fraction scattered between 70% and more than 99% of all Ig-positive cells.

Patients with monoclonal nonsecretory plasma cells. In 6 patients where careful serum and urine analyses showed no evidence for a paraprotein or a Bence Jones protein a monoclonal population of Ig-synthesizing BM cells could be demonstrated

Discussion

Immunologically Normal Individuals

The mean value for Ig-containing BM cells ascertained by cytoplasmic immunofluorescence with FITC-anti Ig was approximately 0.45% of the nucleated cells. As expected from morphological correspondence, this figure agrees with normal values for plasma cells in routine BM smears stained by standard techniques [19]. The percentage distribution of BM cells with intracellular heavy and light chains shown in table I is similar to values reported earlier for BM [4, 15] and comparable to values observed in spleen and tonsils [2, 3, 10, 15]. It is known that molecules of the various Ig classes differ in their rate of catabolism and in the distribution within body compartments [18]. When these differences are considered, the percentage distribution of Ig-containing BM cells corresponds to the relative Ig serum concentrations [4].

Patients with Increased Numbers of Ig-Producing BM Cells of Polyclonal Origin

At the time when BM was obtained from the patients listed in table II the definitive diagnosis was not established and the possibility of an early stage of myeloma or related disorders and particularly of nonsecretory myeloma had to be considered. The percentage distribution of the BM plasma cells with regard to Ig classes and types, however, was clearly polyclonal.

Patients with Increased Numbers of Ig-Producing BM Cells of Monoclonal Origin

Between the magnitude of the M gradient expressed in percent of serum protein and the total number of Ig cells, a linear relationship could be

- 14 TAYLOR, C. R. and MASON, D. Y. The immunohistological detection of intracellular immunoglobulin in formalin-paraffin sections from multiple myeloma and related conditions using the immunoperoxidase technique. *Clin. exp. Immunol.* 18, 417-429 (1974).
- 15 TUNSSON, L. Distribution of immunoglobulin-containing cells in human bone marrow and lymphoid tissue. *Acta. med. scand.* 199- 293-304 (1976).
- 16 UNCC Technical Report Series in WARNER, POTTER, and MITSCHLY Multiple myeloma and related immunoglobulin-producing neoplasms, Geneva, pp.22-25 (1974)
- 17 WALDHENTRICH, J. Diagnosis and treatment of multiple myeloma, pp 15-28 (Grune & Stratton, New York 1970).
- 18 WALDMAN, T. A. and STROMER, W. Metabolism of immunoglobulins. *Prog. Allergy* vol. 13, pp. 1-110 (Karger Basel 1969).
- 19 WILLIAMS, W. J. BEUTLER, E., ESKLEY, A. J. and RISTOLIN, R. W. Hematology pp. 23-29 (Mc Graw-Hill Book Comp. New York 1972).

and laboratory signs of multiple myeloma or Waldenström's macroglobulinemia.

Acknowledgement

We wish to express our gratitude to Miss MONTQUE NEP for her skilful assistance and Dr W HUMANS for his advice and for many helpful discussions.

References

- 1 ARIMO W P and ADAMSON J W. Nonsecretory myeloma. Immunofluorescent demonstration of paraprotein within bone marrow plasma cells. *Cancer* 33 721-728 (1974).
- 2 BEKKER, G M and CORMA, J J. Frequency distribution of α , γ , κ and λ polypeptide chains in human lymphoid tissues. *J. Immun.* 95 246-253 (1965).
- 3 CHIAPPINO, G and FERNIS, B. Demonstration with immunofluorescence of 19S macroglobulins and 7S gammaglobulins in different cells of the human spleen. *Pathol. Microbiol.* 27 8-15 (1964).
- 4 HUMANS, W, SCHUTT H R. E., and HULSTING-HESSELINK, E. An immunofluorescence study on intracellular immunoglobulins in human bone marrow cells. *Ann. N.Y. Acad. Sci.* 177 290-305 (1964).
- 5 HUMANS, W, SCHUTT H R. E., and KLEIN, F. An immunofluorescence procedure for the detection of intracellular immunoglobulins. *Clin. exp. Immunol.* 4 457-472 (1969).
- 6 HUXER, D, PREUD'HOMME, J L., and SELIGSMANN, M. Intracellular monoclonal immunoglobulin in nonsecretory human myeloma. *J. Immun.* 104 263-264 (1970).
- 7 KYLE, R. A. and BAYRD E. D. The monoclonal gammopathies: multiple myeloma and related plasma-cell disorders, pp. 116-135 (Thomas, Springfield 1976).
- 8 MORELL, A, SKVAREL, F, HUMANS, W and SCHERZ, R. Cytoplasmic immunofluorescence of bone marrow plasma cells producing immunoglobulins of the four IgG subclasses. *J. Immun.* 115 579-583 (1975).
- 9 OSEKERMANN E. F and TAKATSUKI, R.. Plasma cell myeloma. Gammaglobulin synthesis and structure. *Medicine, Baltimore* 42 357-384 (1963).
- 10 FERNIS, B. and CHIAPPINO, G. Identification in human lymphoid tissues of cells that produce group 1 and group 2 gammaglobulins. *Immunology* 7 500-506 (1964).
- 11 PREUD'HOMME, J L, HUXER, D, DANON, F., BROUET J C., and SELIGSMANN M. Intracytoplasmic and surface-bound immunoglobulins in 'nonsecretory' and Bence-Jones myeloma. *Clin. exp. Immunol.* 25 428-436 (1976).
- 12 SNAPPER, L. and KAHN A.. Myelomatosis, fundamentals and clinical features, pp. 49-83 (Karger Basel 1971).
- 13 STITES, D. P and WHITEHOUSE, M J.. Evolution of multiple myeloma with non-secreted paraproteins. *Clin. Res.* 23 283A (1975).

- 14 TAYLOR, C. R. and MASON, D. Y. The immunohistological detection of intracellular immunoglobulin in formalin-paraffin sections from multiple myeloma and related conditions using the immunoperoxidase technique. *Clin. exp. Immunol.* 18, 417-429 (1974).
- 15 TUNSSON, L. Distribution of immunoglobulin-containing cells in human bone marrow and lymphoid tissue. *Acta. med. scand.* 199, 293-304 (1976).
- 16 UICC Technical Report Series; in WARNER, POTTER, and MISTALF Multiple myeloma and related immunoglobulin-producing neoplasms, Geneva, pp. 22-25 (1974).
- 17 WALDHENSTRÖM, J. Diagnosis and treatment of multiple myeloma, pp 15-28 (Grune & Stratton, New York 1970).
- 18 WALDMAN, T. A. and STROBER, W. Metabolism of immunoglobulins. *Prog. Allergy* vol. 13, pp. 1-110 (Karger Basel 1969).
- 19 WILLIAMS, W. J. BEUTLER, E., ESKLY, A. J. and RODERS, R. W. Hematology pp. 23-29 (M. Grew-Hill Book Comp. New York 1977).

and laboratory signs of multiple myeloma or Waldenström's macroglobulinemia.

Acknowledgement

We wish to express our gratitude to Miss Monique Nef for her skilful assistance and Dr W HUMANS for his advice and for many helpful discussions.

References

- 1 ABEND, W P and ADAMSON J W.. Nonsecretory myeloma. Immunofluorescent demonstration of paraprotein within bone marrow plasma cells. *Cancer* 33 721-728 (1974).
- 2 BERNIER, G M and CHARRA, J J.. Frequency distribution of α , γ , κ and λ polypeptide chains in human lymphoid tissues. *J Immun.* 93 246-253 (1965).
- 3 CHIAFFINO, G and PERINI, B.. Demonstration with immunofluorescence of 19S macroglobulins and 7S gammaglobulins in different cells of the human spleen. *Pathol. Microbiol.* 27 8-15 (1964).
- 4 HUMANS, W SCHUTT H. R. E., and HULSMAN-HESSELINK, E.. An immunofluorescence study on intracellular immunoglobulins in human bone marrow cells. *Ann. N.Y. Acad. Sci.* 177 290-305 (1964).
- 5 HUMANS, W SCHUTT H. R. E., and KLEIN, F.. An immunofluorescence procedure for the detection of intracellular immunoglobulins. *Clin. exp. Immunol.* 4 457-472 (1969).
- 6 HUXER, D PREUD'HOMME, J L., and SELIGSMAN, M.. Intracellular monoclonal immunoglobulin in nonsecretory human myeloma. *J Immun.* 104 263-264 (1970).
- 7 KYLE, R. A. and BAYRD, E. D.: The monoclonal gammopathies: multiple myeloma and related plasma-cell disorders, pp 116-135 (Thomas, Springfield 1976)
- 8 MORELL, A., SEVARI, F HUMANS, W and SCHERZ, R.. Cytoplasmic immunofluorescence of bone marrow plasma cells producing immunoglobulins of the four IgG subclasses. *J Immun.* 115 579-583 (1975).
- 9 OSKERMANN, E. F and TAKATSUKI, R.. Plasma cell myeloma. Gammaglobulin synthesis and structure. *Medicine, Baltimore* 42 357-384 (1963).
- 10 PERINI, B and CHIAFFINO, G.. Identification in human lymphoid tissues of cells that produce group 1 and group 2 gammaglobulins. *Immunology* 7 500-506 (1964).
- 11 PREUD'HOMME, J L. HUXER, D., DANOFF, F BROUET J C., and SELIGSMAN, M.. Intracytoplasmic and surface-bound immunoglobulins in 'nonsecretory' and Bence-Jones myeloma. *Clin. exp. Immunol.* 25 428-436 (1976)
- 12 SHAPIRO, L. and KAHN A.. Myelomatosis, fundamentals and clinical features, pp. 49-83 (Karger Basel 1971).
- 13 STITES, D P and WHITEHOUSE, M. J.. Evolution of multiple myeloma with non-secreted paraproteins. *Clin. Res.* 23 483A (1975).

allogeneic cells has been used for the evaluation of cellular immunity in a wide range of clinical states and diseases [11, 16, 19, 22]. In normal subjects McIntire and Cole [17] revealed marked variations in lymphocyte blastogenesis. Dionisi *et al.* [8] showed fluctuations in reactivity of lymphocytes from healthy donors and suggested the existence of intrinsic biological cyclicity in the function of these cells. In most clinical studies, however only single determinations of lymphocyte transformation were performed. In a few instances like after renal transplant [5] and in cancer patients before and after various treatments [12] multiple lymphocyte transformation tests were done for monitoring the immune competence.

As to the method for determining the blastogenic transformation, the measurement of radioactive thymidine uptake into DNA of stimulated lymphocytes is described as the most precise one [1]. This response is usually expressed in disintegrations per minute (dpm) either in the presence or in the absence of the mitogen. The calculation of the stimulation index (or transformation index), namely the ratio between the dpm of the stimulated lymphocytes by mitogen and the dpm of spontaneous transformation is a further way to express lymphocyte function [18, 20]. In spite of the frequent use of this index, Coulson *et al.* [7] doubted the justification of using it for denoting objective immunological function. The purpose of this study was to investigate the fluctuations in lymphocyte reactivity according to the frequency of tests performance, as well as the validity of the stimulation index in relation to the dpm values, and whether other methods better expressed the lymphocyte function.

Materials and Methods

All persons investigated were healthy hospital personnel and volunteers. Blood was withdrawn from all individuals for lymphocyte stimulation between 8-9 a.m., according to two time schedules: 9 of the 24 persons denoted as group A were studied twice a week, on Mondays and Fridays. The average period of investigation was 40 days (range 30-60 days). Each individual underwent, during this time, 6-11 lymphocyte transformation tests (LTT). The other 15 persons, comprising group B underwent investigations with long intervals in-between during a mean period of approximately 10 months. Each person had 3-10 LTT. A total of 146 LTT were set up in both groups of individuals with an average of six *in vitro* tests per person.

Lymphocyte Cultures

20 ml blood was drawn into test tubes containing heparin and macrodex (dex tran 70, Pharmacia, Uppsala, Sweden) and mixed. The test tubes were placed at 45° angle at room temperature until erythrocyte sedimentation had taken place (20-

Multiple Lymphocyte Transformation Tests by Phytohemagglutinin in Healthy Individuals

Transient Episodes of Decreased Lymphocyte Blastogenesis

A. BARTAL, T. MEKORI, R. HAASZ, Y. COHEN and E. ROBINSON

The Northern Israel Oncology Center, Rambam Medical Center and
the Abu Khouby School of Medicine, The Technion, Haifa

Key Words: Lymphocyte blastogenesis, PHA, Indices for lymphocyte stimulation evaluation

Abstract 146 serial determinations of spontaneous and phytohemagglutinin-induced lymphocyte transformation as measured by tritiated thymidine incorporation, were performed in 24 healthy individuals. 9 persons were studied intensively during a mean period of 40 days and the other 15 underwent random investigations during a period up to 10 months. Transient episodes of significant decreased lymphocyte transformation were revealed in 14 persons (58.4%) with regard to the spontaneous blastogenesis and in 11 subjects (45.8%) with regard to PHA-induced reactivity. The occurrence of the decreased values in PHA responsiveness was significantly higher in the intensively studied group compared to the randomly investigated ones ($p = 0.001$). The probability of detecting one low PHA related response in a normal subject was 11%. In only half of the cultures with low spontaneous blastogenic response did a simultaneous decrease in PHA-induced lymphocyte transformation occur. In addition to the currently used stimulation index, a new index termed the Blastogenic Cumulative Index is proposed. It is defined as the sum of the logarithms of the dpm values of the spontaneous and the PHA induced lymphocyte transformation. The CI is suggested to describe lymphocyte blastogenic function more accurately and due to its simplicity warrants further study. Other indices for lymphocyte blastogenesis determination are discussed.

Introduction

The property of lymphocytes to undergo transformation *in vitro* into large lymphoblastoid cells in response to mitogens, bacterial antigens and

allogeneic cells has been used for the evaluation of cellular immunity in a wide range of clinical states and diseases [11, 16, 19, 22]. In normal subjects McINTIRE and COLE [17] revealed marked variations in lymphocyte blastogenesis. DIENNOT *et al.* [8] showed fluctuations in reactivity of lymphocytes from healthy donors and suggested the existence of intrinsic biological cyclicity in the function of these cells. In most clinical studies, however, only single determinations of lymphocyte transformation were performed. In a few instances like after renal transplant [5] and in cancer patients before and after various treatments [12] multiple lymphocyte transformation tests were done for monitoring the immune competence.

As to the method for determining the blastogenic transformation, the measurement of radioactive thymidine uptake into DNA of stimulated lymphocytes is described as the most precise one [1]. This response is usually expressed in disintegrations per minute (dpm), either in the presence or in the absence of the mitogen. The calculation of the stimulation index (or transformation index), namely the ratio between the dpm of the stimulated lymphocytes by mitogen and the dpm of spontaneous transformation is a further way to express lymphocyte function [18, 20]. In spite of the frequent use of this index, COULSON *et al.* [7] doubted the justification of using it for denoting objective immunological function. The purpose of this study was to investigate the fluctuations in lymphocyte reactivity according to the frequency of tests performance, as well as the validity of the stimulation index in relation to the dpm values, and whether other methods better expressed the lymphocyte function.

Materials and Methods

All persons investigated were healthy hospital personnel and volunteers. Blood was withdrawn from all individuals for lymphocyte stimulation between 8-9 a.m., according to two time schedules: 9 of the 24 persons denoted as group A were studied twice a week, on Mondays and Fridays. The average period of investigation was 40 days (range 30-60 days). Each individual underwent, during this time, 6-11 lymphocyte transformation tests (LTT). The other 15 persons, comprising group B, underwent investigations with long intervals in-between during a mean period of approximately 111 months. Each person had 3-10 LTT. A total of 146 LTT were set up in both groups of individuals with an average of six to five tests per person.

Lymphocyte Cultures

20 ml blood was drawn into test tubes containing heparin and macrodex (dextran 70, Pharmacia, Uppsala, Sweden) and mixed. The test tubes were placed at 45° angle at room temperature until erythrocyte sedimentation had taken place (20-

Multiple Lymphocyte Transformation Tests by Phytohemagglutinin in Healthy Individuals

Transient Episodes of Decreased Lymphocyte Blastogenesis

A BARTAL, T MEKORI, R. HAASZ, Y COHEN and E. ROBINSON

The Northern Israel Oncology Center, Rambam Medical Center and
the Aba Khoushy School of Medicine, The Technion, Haifa

Key Words Lymphocyte blastogenesis · PHA Indices for lymphocyte stimulation evaluation

Abstract 146 serial determinations of spontaneous and phytohemagglutinin-induced lymphocyte transformation as measured by tritiated thymidine incorporation, were performed in 24 healthy individuals. 9 persons were studied intensively during a mean period of 40 days and the other 15 underwent random investigations during a period up to 10 months. Transient episodes of significant decreased lymphocyte transformation were revealed in 14 persons (58.4%) with regard to the spontaneous blastogenesis and in 11 subjects (45.8%) with regard to PHA induced reactivity. The occurrence of the decreased values in PHA responsiveness was significantly higher in the intensively studied group compared to the randomly investigated one ($p = 0.001$). The probability of detecting one low PHA-related response in a normal subject was 11%. In only half of the cultures with low spontaneous blastogenic response did a simultaneous decrease in PHA induced lymphocyte transformation occur. In addition to the currently used stimulation index, a new index termed the Blastogenic Cumulative Index is proposed. It is defined as the sum of the logarithms of the dpm values of the spontaneous and the PHA induced lymphocyte transformation. The CI is suggested to describe lymphocyte blastogenic function more accurately and due to its simplicity warrants further study. Other indices for lymphocyte blastogenesis determination are discussed.

Introduction

The property of lymphocytes to undergo transformation *in vitro* into large lymphoblastoid cells in response to mitogens, bacterial antigens and

Results

The results of the spontaneous lymphocyte transformation tests performed in the 24 individuals are shown in table I. Values were found to be scattered over a wide range of activity in all subjects. In 14 individuals (58.4 %) 1-5 episodes per person of decreased spontaneous reactivity were detected, amounting to a total of 21 (14.3%) out of the 146 tests performed. The mean SP-LT was 1,569 dpm (SE 467 dpm) and the range 35-48,875 dpm. No significant differences were observed between

Table I Spontaneous and PHA-induced lymphocyte transformation in 24 healthy individuals undergoing multiple *in vitro* testing

	Group A	%	Group B	%	Total	%
Number of subjects	9		15		24	
Number of subjects with SP-LT < 200 dpm	4 (1-5) ^a		10 (1-2) ^a		14	
Number of cultures with SP-LT						
< 200 dpm	9	13.6	12	15	21	14.3
200-1,000 dpm	34	51.5	44	55	78	51.4
> 1,000 dpm	23	34.8	24	30	47	32.2
Total	66	100	80	100	146	100
Mean dpm	1,919		1,419		1,569	
Median dpm	600		700		700	
Number of subjects with PHA-LT						
< 10,000 dpm	6 (1-4) ^a		5 (1) ^a		11	
Number of cultures with PHA-LT						
< 10,000 dpm	12	18.1	5	6.2	17	11.6
10,000-100,000 dpm	17	25.7	11	13.7	28	19.2
> 100,000 dpm	37	56.1	23	28.7	60	41.1
Total	66	100	80	100	146	100
Mean dpm	184,077 ^a		82,783		123,667	
Median dpm	190,000		68,000		82,000	

Number of episodes of decreased lymphocyte transformation by PHA.

p = 0.02.

30 min) The supernatant, the leukocyte-rich plasma, was transferred to sterile test tubes and centrifuged at 700 rpm for 7 min. The supernatant was discarded and the cells were suspended in 10 ml medium 199 (M 199). After centrifugation the precipitate was suspended and used for culture. Cultures of leukocytes containing 1×10^6 lymphocytes in 2 ml M 199 containing 15% AB serum were prepared in tubes. The medium was supplemented with antibiotics 100 U penicillin and 100 μ g streptomycin/ml. Half the cultures were set up with 500 μ g (0.05 ml) PHA (Wellcome Research Laboratories, Beckenham, England) while the other half served as unstimulated controls. The cultures were set up in triplicate and incubated at 37 °C with 5% CO₂ atmosphere for 72 h. Lymphocyte transformation was tested by the incorporation of ³H tritiated thymidine indicating DNA synthesis during the last 17 h of incubation. At the end of the labelling period, the cultures were harvested by washing the cells in ice-cold saline. The nuclear protein was precipitated by 2 ml of ice-cold 5% trichloroacetic acid. The final precipitate was resuspended and passed through a filter disc (Whatman glass paper CF/A). The dried filters were transferred into bottles containing scintillation solution and the radioactivity was measured in a Packard liquid scintillation counter.

Lymphocyte Blastogenesis Evaluation

The lymphocyte blastogenesis response was expressed in each of the following methods:

(1) Disintegration per minute (dpm) The mean dpm of the 3 test tubes and the standard error were calculated for both the spontaneous lymphocyte transformation (SP-LT) and the phytohemagglutinin-induced transformation (PHA LT). SP-LT values were defined to be low under 200 dpm and PHA LT when below 10,000 dpm.

(2) Stimulation Index (SI) The SI of the lymphocyte responses was calculated by dividing the dpm of lymphocytes stimulated by PHA by the dpm of control cultures. The data was analyzed twice, considering a low SI when below 50 (the value obtained when dividing 10,000 by 200 dpm) or below 20 as previously used [2].

(3) Blastogenic cumulative index, or CI A new index for evaluation of the lymphocyte blastogenesis function is proposed in this study defined as the logarithmic sum of the spontaneous transformation in dpm, and the dpm obtained after PHA stimulation, as following: $\log \text{dpm (SP LT)} + \log \text{dpm (PHA LT)} = \text{CI}$. The cumulative index was considered low at less than 6.3 based on the formula $\log 200 \text{ dpm} + \log 10,000 \text{ dpm}$ resulting in this figure.

Statistical Analysis

Statistical analysis was carried out separately for spontaneous and PHA-induced lymphocyte transformation data. Since the plot of the dpm values of both these responses on an arithmetic scale showed a widely spread distribution, logarithmic functions were used.

These functions provided closer approximation to normal distribution as demonstrated by a straight line when plotting the responses on a probability cumulative paper [23]. For both types of responses the Wilcoxon Two Sample Rank Test was used in order to check whether randomly and intensively investigated groups possess the same distribution. Confidence intervals for the probability of a response being low (e.g. below 10,000 dpm) were obtained using the nonparametric method [3].

Table II The distribution of the SI of the 146 LTT

SI	Number of cultures	%
< 20	23	15.7
< 50	41	28.1
50-100	21	14.4
100-200	39	26.7
200-500	35	24.0
500-1,000	10	6.8
Total	146	100.0

Table III The distribution of the CI of the 146 LTT

CI	Number of cultures	%
Below 6.3	19	13
6.4-7.9	67	45.9
Above 8.0	60	41.1

Table IV The level of the SI as compared to that of CIs in the 24 individuals

Number of persons	SI	CI
4	low	high
5	high	low
9	low	low
6	high	high

Values below SI = 20.

The Stimulation and Cumulative Indices

The stimulation indices obtained in the 146 LTT were found to spread over a wide range, from 0.93 to 1041 mean 170 and median 128 (table II). Of the 24 persons tested, 13 (54.1%) had at least one episode of SI below 20 and 17 (70.8%) had at least one value less than 50. Only 2 individuals had SI values above 100 with no episode beneath this level.

A much narrower range of values was obtained when calculating the CI being from 4.2 to 10.2, mean 7.4 and median 7.5 (table III). 14 individuals (58.3%) had a CI below 6.3 at least once. It is of interest to note

the pattern of responses in groups A and B both showing a log-normal distribution.

The wide range of lymphocyte responses after stimulation with PHA is demonstrated in table I. In 11 of the 24 persons (45.8%) at least one determination was below 10 000 dpm. The mean values of all determinations was 123 667 (SE 10 148 dpm) and range 156–393 719 dpm. The probability of a healthy individual to have a decreased lymphocyte blastogenic response to PHA was 11%. The probability of obtaining two successive low lymphocyte responses in a normal individual was much lower being 1%. Episodes of decreased function were significantly more frequent in the intensively studied group than in the randomly studied one, the probability being 18 vs. 6%, respectively ($p = 0.001$). The magnitude of lymphocyte responses was significantly higher in the intensively investigated than in the randomly studied group ($p = 0.02$).

The analysis of the PHA induced lymphocyte responses in the 146 cultures showed absence of normal distribution. Splitting the combined group of responses according to the schedule of investigation revealed the existence of log normal distribution in the group B data (fig. 1B) but not in the group A responses (fig. 1A). Correlation between decreased spontaneous lymphocyte blastogenesis and decreased PHA induced transformation data was detected only in about half of the cultures.

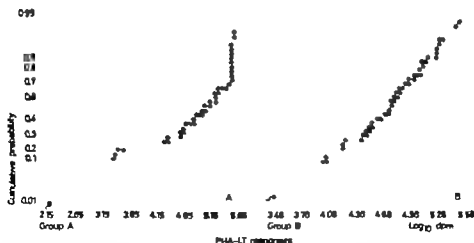


Fig 1 PHA lymphocyte transformation responses plotted against cumulative probability. A Log-responses of group A individuals showing a highly curved line. B The plot of log-responses of group B individuals against probability closely approximates a straight line.

Table V The interrelationship between SP-LT PHA-LT CI and SI in the 146 lymphocyte cultures

	Number of cultures			
	PHA-LT < 10,000 dpm SP-LT < 200	PHA-LT < 10,000 dpm SP-LT > 200	PHA-LT > 10,000 dpm SP-LT > 200	PHA-LT > 10,000 dpm SP-LT < 200
SI > 50	-	-	91	5
CI > 6.3			(62.3%)	5 (3.4%)
SI < 50	-	3	28	-
CI > 6.3		(2.0%)	(19.1%)	
SI < 50	7	3		
CI < 6.3	(4.8%)	(2.0%)		
SI > 50	4			5
CI < 6.3	(2.7%)			(3.4%)

together the CI better represented the dpm values of the SP-LT and PHA-LT than the SI.

Sequential studies of lymphocyte blastogenesis as monitored in one of the subjects are illustrated in figure 3. Cumulative indices fluctuated very similarly to changes in SP-LT and PHA-LT whereas SI showed a bizarre pattern with no correlation to dpm variations.

Discussion

The present study has confirmed previous reports showing that lymphocyte blastogenesis in normal subjects, whether spontaneous or induced by PHA, is not a constant high value but is distributed over a wide range of activity [8, 17]. Fluctuations in the immune competence has also been outlined by GILLESPIE and BARTH [10] using the allograft rejection technique. Our study has shown that the probability of a healthy individual to have a solitary episode of decreased lymphocyte reactivity to PHA is 11%. However, the probability of obtaining two successive responses with decreased function in the same person was much lower being 1%. The schedule of immune investigation was revealed to play an important role in the determination of the PHA-induced lymphocyte response pattern. Log-normal distribution was demonstrated to occur only with the random monitoring and not in the intensively studied group. The reason for this

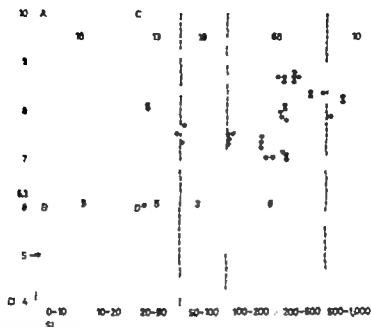


Fig 2 The relation between the cumulative and the stimulation indices as found in 146 *in vitro* lymphocyte transformation tests.

that individuals could be shown to have low blastogenic function according to one index and still be competent according to the other (table IV fig 2) Of 23 tests with SI below 20 only 5 tests (21.7%) showed a low CI the remaining 18 tests having a high CI (fig. 2, area A) This implies that both DPM of spontaneous and PHA LT were high and only their ratio was low Of the 123 cultures with SI above 20, in 14 tests (11.4%), a CI was found to be under 6.3 (fig. 2, area D)

The overall absence of correlation between the SI and CI amounted to 32 LTT of the 146 performed (22.0%) When considering SI 50 as the lower border the overall incidence of noncorrelation amounted to 27.4% (40 tests of the 146)

The relationship between the SP LT PHA LT SI and CI is demonstrated in table V In 91 of the tests (62.3%) all four figures were high, and in 7 other tests (4.8%) all were low Isolated decrease in SI was observed in 31 determinations (21.2%) whereas in no case could the CI be shown to be low by itself Other modes of association are illustrated as well, such as the occurrence of low PHA LT with high SP LT values yielding low SI and high CI or high PHA LT responses with low SP LT responses resulting in high SI whereas the cumulative index was low A)

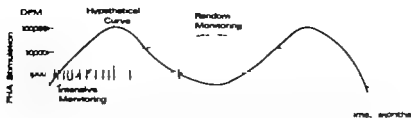


Fig 4 A hypothetical curve demonstrating cyclical fluctuations of PHA-induced lymphocyte transformation responses plotted against time according to the schedule of investigations in this study. Intensive monitoring may presumably detect responses belonging to a part of a cycle while upon random and scarce testing one detects incidental lymphocyte responses of various cycles.

We propose in this study the use of a new index for the evaluation of lymphocyte blastogenesis to be called the Blastogenic Cumulative Index or CI. This index is by its definition the addition of two lymphocytic competences. The irrationality of using the stimulation index so often used for evaluating lymphocyte function [18, 20] has been outlined before [7]. This study has further demonstrated the frequently occurring pitfalls when using this index alone. In 27.4% of the tests no correlation could be found between the magnitude of the dpm values of stimulated and unstimulated cultures and their ratio. This is due to the fact that spontaneous transformation is inversely correlated with the SI. Whenever the spontaneous transformation will increase, the SI will decrease even if the PHA-induced transformation is high, and vice versa. This does not occur with the CI being in direct relationship with the spontaneous and PHA-related transformation and due to the logarithmic functions, it expresses more accurately these two competences.

Moreover CARTER *et al.* [6] reported diurnal variations in spontaneous blastogenesis of lymphocytes from normal persons studied at 2-hourly intervals for 24 h. These small fluctuations normally existing in the spontaneous blastogenesis may cause big changes in the SI without expressing meaningful biological alteration in the cells themselves. Spontaneous and antigen-induced transformation seem to occur through different biological mechanisms, the former being triggered by an endogenous pathway in the cell nucleus, whereas the latter by an exogenous activation through membrane changes, thereafter gene activity and cell mitosis [14]. It seems therefore justified to add these two capacities, independent one

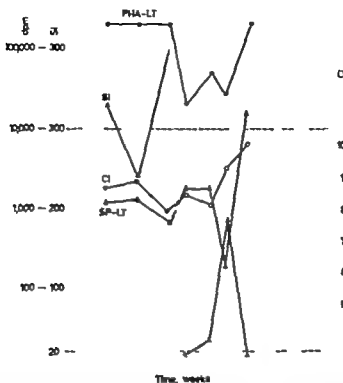


Fig 3 Changes in spontaneous and PHA-induced lymphocyte transformation compared to the simultaneous fluctuations in the CI and SI in 1 healthy individual undergoing sequential studies. Note the bizarre pattern of the SI curve, whereas the CI curve is similar to the dpm curves.

observation is unknown but if one presumes the existence of cyclical fluctuations in lymphocyte blastogenic capacity then with long interval monitoring a summation of several cycles will occur yielding a log-normal distribution. With intensive investigation one may detect responses belonging to a part of one cycle only and thus normal distribution is not achieved (fig. 4)

Variations in the lymphocyte reactivity may be explained by other causes than the biological cyclicity hypothesis. The events that occur in the test culture are known to be complex. Variations may thus occur due to different binding capacity of PHA by the lymphocytes [9] by serum factors present which influence this binding or effect cell viability in the culture, and by differences in purification of cells and possible changes in lymphocyte populations in the peripheral blood at different periods [17, 21]. Presence of occult infections in the studied individuals as well as periodontal disease [15] may affect the pattern of their responses.

use of the CI may prove useful in clinical investigations and should therefore be in aid in future studies in evaluation of lymphocyte stimulation. It is also suggested that multiple LTT should be performed in the future since single low values of lymphocyte reactivity with or without mitogen do not exclude normal function. An immunological profile consisting of at least 2 successive LTT is mandatory. The cyclicity of lymphocyte responses in normal and diseased states should be further investigated.

Acknowledgements

We are grateful to Dr PETER KOVAT, Department of Statistics, Technion, Haifa, for statistical analysis; Mrs RUTH SEGAL for excellent technical assistance and Mrs GRETTEL MEROM for typing the manuscript. This work was supported in part by donation in memory of the late LUKE SUTERMAN administered by the Office of the Administrator General, Ministry of Justice and the Medical Research Fund under the sponsorship of the Ministry of Health, Israel. E. ROSENBOOM is an associated professor and established investigator of the Chief Scientists' Bureau, Ministry of Health, Israel.

References

- 1 BACH, F. H. Measurement of delayed hypersensitivity in KAGAN and STERN Immunologic incompetence, pp. 137-146 (Year Book, Chicago 1971).
- 2 BARTAL, A., HAALEY, R., STEDER, M., COHEN, Y. and ROSENBOOM, E. Cellular immunity in patients with multiple primary tumors. *Cancer Immunol. Immunother.* 2, 195-199 (1977).
- 3 BROWNE, K. A. Statistical theory and methodology in science and engineering, pp. 150-157 (Wiley New York 1967).
- 4 CAMPBELL, A. C., HERBERT, P., HARRISON, B., HOLLINGSWORTH, P. M., SCHUCKER, J., and MACLEODMAN, I. C. M. Effects of anti cancer agents on immunological status. *Br J Cancer* 28 suppl. 1, pp. 254-261 (1973).
- 5 COMELAND, D., RASTED, P., STEWART, T. and HARRIS, J. The use of *in vitro* lymphocyte responsiveness to specific mitogenic agents in the immunological monitoring of human renal allograft recipients. *Tissue Antigens* 4, 106-114 (1974).
- 6 CARTER, J. B., BARR, G. D., LEVIN, A. S., BYERS, V. S., POWELL, B., FIDELLERHO, H. H., and GERMAN, D. I. Standardization of tissue culture conditions for spontaneous thymidine-22 $^{\circ}$ C incorporation by unstimulated normal human peripheral lymphocytes. Circadian rhythm of DNA synthesis. *J Allergy clin. Immunol.* 56, 191-205 (1975).
- 7 COULSON, A. S., MACMILLAN, F., GRIFF, R. B., STEINBERG, E. B., DOMO, E., and SHIMWY, Y. N. E. Lymphocyte tissue culture studies on human heart transplant recipients. *Transplantation* 18, 409-415 (1974).
- 8 DICKSON, R., ZONTA, A., ALBERTARIO, F., GALIAZZI, R., and RYLANDSON, G.

Table VI Comparison between the features of the SI and the CI

CI	SI
1) The sum of the logarithms of dpm values of spontaneous and PHA related lymphocyte responses	1) the ratio between the dpm values of the PHA-induced lymphocyte transformation and the spontaneous blastogenesis
2) The CI is directly correlated to spontaneous blastogenesis	2) the SI is inversely correlated to the spontaneous blastogenesis
3) The sum of two related independent biological responses	3) a ratio of two biological processes that are probably independent but not synchronous
4) The CI has a narrow range of normal values (6.3-10.0), and therefore is simple for current use	4) The normal values of the SI spread over a wide range (20-1,000)
5) A rise in the CI signifies a rise in lymphocyte blastogenic function	5) rise in the SI does not necessarily represent increase in lymphocyte function, it may represent merely a decrease in SP LT
6) It indicates the total lymphocyte blastogenic competence represented by both the dpm values of spontaneous and mitogen-induced blastogenesis	6) It indicates how much more the mitogen stimulates the lymphocytes than without it
7) The logarithmic transformation of the dpm values has been shown to be superior to the arithmetic figures in statistical analysis	7) statistical analysis of dpm values not converted into logarithms do not show normal distribution

of the other rather than use their ratio. The advantages of the CI are described in table VI. It is characterized by its simplicity, the narrow range of normal values accomplished through the logarithmic converted dpm values described by ZIGLER *et al.* [23] to be superior upon statistical analysis to the arithmetic values of transformation. The CI is thus suggested to represent better the blastogenic function. It is not yet clear whether there is any correlation between a decrease in spontaneous transformation and immunodeficiency since to the best of our knowledge no study entangled this issue. Other indices for blastogenesis evaluation have been suggested by various authors. CAMPBELL [4] used the mitogenic capacity index calculated as the \log_{10} (PHA response \times lymphocyte count/ml.) HOSKING *et al.* [13] using multiple doses of PHA suggested a complex index called the PHA-dose response ratio namely the ratio of \log (cpm, 200-0 μ g PHA) to \log (cpm 20-0 μ g PHA). It seems to us that the

Ethnic and Clinical Aspects of Chronic Lymphocytic Leukemia in Israel

A Survey on 288 Patients

A. BARTAL, Z. BENTWICH, N. MANNY and G. IZAK²

Department of Hematology Hebrew University Hadassah Medical School,
Jerusalem

Key Words. Chronic lymphocytic leukemia Ethnicity Multiple primary neoplasms Immunoglobulins Hemolytic anemia

Abstract The ethnic distribution of 288 patients with chronic lymphocytic leukemia (CLL) from five medical centers in Israel during the decade 1960-1970 was investigated as well as other features of the disease. 80.5% of the patients were of East European descent, 14.5% originated from Asia and Africa and 5% were born in Israel. Based on the ethnic distribution of the elderly population of Israel during the above period, it is suggested that CLL occurs more frequently in European-born Jews than in Asian and African Jews. A low occurrence of Coombs positive hemolytic anemia was revealed, amounting to 7.2% of the patients. Of 68 patients who underwent serum immunoglobulin studies, up to 74% exhibited deficiency of at least one type of immunoglobulin. Low levels of IgA were encountered in 61.2%, IgM in 51.5% and IgG in 29.5% of the patients. 26 of 206 patients with CLL (12.5%), all of East European origin, had an additional primary malignant tumor: cancer of the skin and breast being the most frequent associated malignancies. In 6 patients of the 26, two additional neoplasms were diagnosed. Hypogammaglobulinemia was more frequent in patients with additional primary malignant tumors and in patients with advanced disease. The possible causes for the ethnic distribution of CLL in Israel as well as of other tumors are discussed.

Introduction

During the 130 years since it was first described [1], chronic lymphocytic leukemia (CLL) has become known as a relatively benign neoplastic

This study is part of the MD thesis delivered to the Hebrew University Hadassah Medical School, Jerusalem.

G. I. is an established investigator of the Ministry of Health, Israel

- Cyclic variation in the response of lymphocytes to phytohemagglutinin in healthy individuals. *Transplantation* 16: 550-557 (1973).
- 9 FORDYKE, D. R.. Quantitative nucleic acid changes during phytohemagglutinin induced lymphocyte transformation *in vitro* dependence of the response on phytohemagglutinin-serum ratio. *Biochem. J* 105 679-684 (1967).
- 10 GILLESPIE, Y. G. and BARTH, R. F.. Cyclic variations in cell mediated immunity to skin allografts detected by the technetium 99M microcytotoxicity assay. *Cell. Immunol.* 13 472-483 (1974).
- 11 HARRIS, J. and COVELAND, D.. Impaired immunoresponsiveness in tumor patients. *Ann. N.Y. Acad. Sci.* 230 56-85 (1974).
- 12 HERSH, E. M., GUTTERMAN, J. U., MAVLIOT, G. M., MCCREDIE, L. B., BURGESS, M. A., MATTHEWS, A., and FREIREICH, E. J.. Serial studies of immunocompetence of patients undergoing chemotherapy for acute leukemia. *J. clin. Invest.* 54 401-408 (1974).
- 13 HOSKING, C. S., FITZGERALD, M. G. and SIMONS, M. J.. Quantified deficiency of lymphocyte response to phytohemagglutinin in immune deficiency diseases. *Clin. exp. Immunol.* 9 467-476 (1971).
- 14 LEVY, R. and KAPLAN, H. S.. Impaired lymphocyte function in untreated Hodgkin's disease. *New Engl. J. Med.* 290 181-186 (1974).
- 15 MACKLER, B. P., ALTMAN, L. C., WAHL, S., ROSENSTREICH, D. L., OPPENHEIM, J. J. and MERGENTHAU, S. E.. Blastogenesis and lymphokine synthesis by T and B lymphocytes from patients with periodontal disease. *Infect. Immunity* 10 844-849 (1974).
- 16 MANGI, R. J., NIEDERMAN, J. C., KELLERER, J. E., DWYER, J. M., EVANS, A. S., and KANTO, F. S.. Depression of cell mediated immunity during acute infectious mononucleosis. *New Engl. J. Med.* 291 1149-1153 (1974).
- 17 MCINTYRE, O. R. and COLE, A. F.. Variation in the response of normal lymphocytes to PHA. *Int. Archs Allergy appl. Immun.* 35 105-118 (1969).
- 18 MEKORI, C. and HAN, T.. Immune response in bladder cancer. *J. Urol.* 111 170-172 (1974).
- 19 PORTILO, D. T., HALLGREN, H. M., and YIVIS, B. J.. Depressed maternal lymphocyte response to PHA in human pregnancy. *Lancet* i 769-771 (1972).
- 20 REIS, J. C., RASIO, J. L., WILSON, H. E., MINTON, J. P. and DODD, M. C.. Cellular immunity in neoplasia. Antigen and mitogen responses in patients with bronchogenic carcinoma. *Cancer* 36 2010-2015 (1975).
- 21 SIMONS, M. J., FOWLER, R., and FITZGERALD, M. G.. The reactivity of human small lymphocytes in culture. The effects of concentration and duration of exposure to phytohemagglutinin. *Cell Tiss. Kinet.* 2 1-9 (1969).
- 22 SOCU-FOCA, N., BUDA, J. A., THOM, T. and REEMTSMA, K.. Impaired responsiveness of lymphocytes in patients with systemic lupus erythematosus. *Clin. exp. Immunol.* 18 293-301 (1974).
- 23 ZIEGLER, J. B., HANSEN, F. J., DAVIES, W. A., and PENNY, R.. The PHA dose response curve. Validation of the use of logarithmic graph paper by computer analysis results. *J. Immun.* 113 2035-2039 (1974).

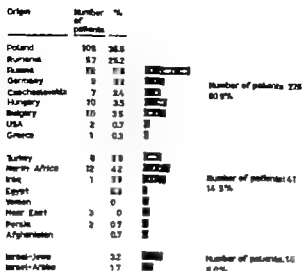


Fig. 1 Ethnic distribution in 281 CLL patients with well-documented origin.

migrated from Africa, the Near East and Far Eastern countries (fig. 1). Furthermore, over 63% of all CLL patients consisted of immigrants from Poland (38.5%) and from Rumania (25.2%). These observations acquire particular significance, in view of the fact that during the decade under study the ethnic distribution of the Israeli population was 67.6% European and 28.1% Near Eastern, Far Eastern and African [14], with a similar distribution of hospitalized patients during the same period (table I). It is of interest to note that 9 of the 14 patients born in Israel were Jews, all of European descent and the remaining 5 patients were of arabic origin.

The bulk of the patients were in their 5th and 6th decade of life at the time of diagnosis (fig. 2). The mean age of disease appearance in patients originating from Asia and Africa was significantly lower than in those born in Europe, 57.5 and 62.5 years, respectively ($p < 0.05$), this being even more prominent in Asia and Africa-born females compared to females from Europe with the disease ($p < 0.01$). The male to female ratio was 4.3 in the European descent patients, whereas in Asia- and Africa-born patients the obtained ratio was 1.2 thus showing female predominance.

disease of the lymphatic system. Despite the substantial amount of accumulated information on the extreme variations in the course of the disease, on its familial occurrence [10] its frequent association with anomalies of the immune system [15] and concerning possible environmental influence, the etiology and pathogenesis of CLL remain obscure [8]. The incidence of CLL was reported to be low in Far Eastern, African and South American populations and high among Caucasians [27-32]. The Jewish population in various countries was also reported to have a high incidence of the disease [16-21]. These observations drew attention to the racial and ethnic distribution of this disease in the understanding of its etiology. As the population of Israel consists of a large number of ethnic groups originating from many parts of the world it seemed of interest to study the prevalence of CLL among them. Such a study on 288 CLL patients is described in the following communication.

Patients and Methods

288 patients diagnosed as suffering from CLL in five medical centers in Israel (Beilinson Medical Center Beer-Sheba Medical Center Hadassah Medical Center of Jerusalem, Rambam Medical Center and Haim Sheba Medical Center Tel Hashomer) during the decade 1960-1970 were studied. Only regular inhabitants of Israel were eligible for the study. The clinical charts at the hematology clinics and hospital medical archives were reviewed. Complete details of individual profiles including age, sex, origin, initial white blood cell count with degree of lymphocytosis, direct and indirect Coombs test, serum immunoglobulin levels, duration of disease and other associated diagnoses were recorded. The criteria for the diagnosis of CLL were: a peripheral blood leukocyte count of above $11,000/\text{mm}^3$ with at least 60% lymphocytosis, and diffuse lymphocytic infiltration of the bone marrow persisting beyond a period of 6 months.

Serum immunoglobulin levels of IgG, IgA and IgM were determined in 88 patients by the radial immunodiffusion technique in agar [17] and the values of Dacie were considered as normal [3]. Treatment consisted of corticosteroids and Leukeran and was instituted only in patients who developed anemia, fever or other systemic manifestations interfering severely with the patients' life.

Student's *t* test was used to assess the statistical significance of differences observed between patients with different ethnic origin.

Results

The distribution of the patients according to their ethnic origin revealed that over 80% of them came from Europe while less than 15% im

Table II Type and occurrence of neoplasms in 207 patients with CLL

Malignant neoplasms			Benign neoplasms		
type	n	%	type	n	%
Skin	10	4.8	myoma	7	3.3
Breast	6	2.9	Kaposi sarcoma	3	1.4
Colon	2	0.9	lipoma	2	0.9
Rectum	2	0.9	breast	2	0.9
Polycythemia vera	2	0.9	neurofibroma	1	0.4
Stomach	1	0.4	thymoma	1	0.4
Uterus	1	0.4			
Hodgkin's	1	0.4			
Lung	1	0.4			
Larynx	(1)				
Kidney	(1)				
Prostate	(1)				
Gumt cell TU of jaw	(1)				
Total	26	12.5		16	7.7

Table III. Type of malignant tumors in CLL patients with more than one additional neoplasm

Skin - multiple basal or SQ cell CA	3
Colon and kidney	1
Larynx and skin	1
Prostate and skin	1
Total (patients)	6

years (mean 8 years), while in the remaining 9 patients both neoplastic processes were noted to appear simultaneously. In 6 of these 26 patients, a third malignant neoplasia was diagnosed subsequently (table III), skin and breast cancer being the most frequent associated tumors. A positive family history of neoplastic disease occurrence was obtained in 5 of these latter patients. In one of these families, all 3 surviving members belonging to 3 generations suffered of CLL (fig. 3).

Coombs positive hemolytic disease was found in 15 of 204 patients (7.2%) in whom this test was monitored. All these patients but 3 were from Eastern Europe, their age was between 50 and 80 years and the

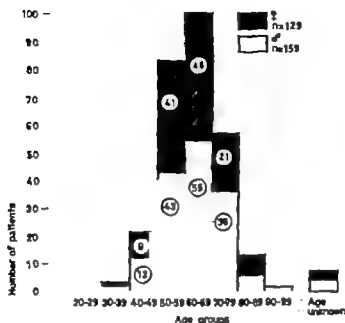


Fig 2 Age and sex distribution in the 288 CLL patients.

Table 1 Ethnic distribution (percent) of the elderly Israeli population between 1960 and 1969 and during 1970 [14] and that of the Hadassah Medical Center hospitalized patients during 1970 as compared to the current study in CLL patients

Origin	Israeli population above age 65 (1960-1969)	Israeli population above age 45 during 1970	Hadassah patients above age 45 during 1970	CLL patients
Europe	67.5	65.6	55.0	80.5
Asia and Africa	28.2	30.0	27.5	14.5
Israel	4.3	4.4	16.0	5.0

One additional tumor was found in 42 (20.2%) of 206 patients with CLL in whom reliable documentation was available. Of these, the neoplasia was malignant in 26 (12.5%) while in 7.7% the tumor was benign (table II). All patients with an additional malignant tumor were of East European origin: 15 of them were males and 11 females. In 8 patients the second tumor was observed during the follow up examination 1-7 years after the CLL was diagnosed (mean 4.3 years). In 9 patients the diagnosis of the malignant tumor preceded the evolution of CLL by between 3-22

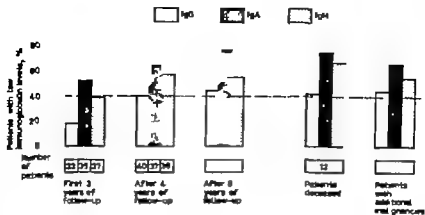


Fig 4 Level of serum immunoglobulins in 68 CLL patients repeatedly performed on several occasions during their disease.

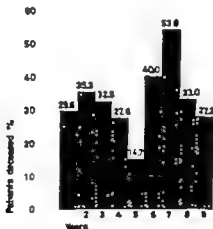


Fig 5 Mortality of patients according to years of follow-up.

There seemed to be a bimodal pattern in the mortality of the patients of European descent only the first peak occurring 2-3 years and the second 6-7 years after diagnosis was established (fig. 5). The overall mean survival was 4 years, the 5-year survival 57.5% 23.6% were alive after 8

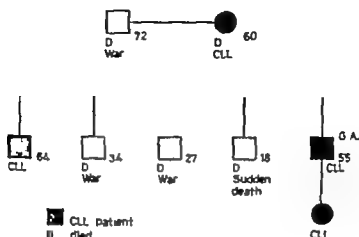


Fig 3 Pedigree showing the appearance of CLL in a family

Table IV Level of serum immunoglobulins in 68 CLL patients

	Total number of patients	High		Normal		Low	
		n		n	%	n	%
IgG	68	3	4.4	45	66.2	20	29.4
IgA	67	1	1.5	25	37.3	41	61.2
IgM	68	6	8.8	27	39.7	35	51.5

male to female ratio was 3:2. The positive Coombs test was found in most patients shortly after CLL diagnosis; in only 2 patients the positive test appeared after 3 and 4 years of follow up. 2 patients with autoimmune hemolytic anemia had further primary malignancies in addition to CLL, one with rectal cancer and the other giant cell tumor of the mandible and basal cell carcinoma of the forehead.

The serum immunoglobulin concentrations estimated in 68 patients revealed low IgG levels in 29.4%, low IgA levels in 61.2% and low levels of IgM in 51.5% of them (table IV). In 74.5% of the patients at least 1 of the 3 above immunoglobulins was below the normal range. The values tended to decrease during the course of the disease (fig 4). Particularly low immunoglobulin concentrations were more frequent in patients shortly before death (83.5%) and in those afflicted with additional neoplasia (78%) (fig. 4).

These findings are in accordance with those reported by LEONCINI *et al.* [15]. It is worthwhile noting the discrepancy between the high incidence of low levels of serum IgA and the relative low incidence of low IgG levels. CLL is reported to be a B cell proliferation [2] and it could have been expected that both IgG and IgA should be similarly depressed, which was not the case in this study.

CLL patients carrying an additional tumor had the highest incidence of low serum immunoglobulin levels. Whether this phenomenon was secondary to the widespread malignancies or may have played a causative role in their evolution, cannot be stated on the basis of the data available at present. It is, however, difficult to reconcile these findings with the relatively low incidence of autoimmune hemolytic anemia associated with CLL in Israeli patients. Coombs positive hemolytic anemia was reported to evolve in 20-36% of CLL patients [7, 20, 22, 25]. SCOTT [24] reported a much lower incidence of hemolytic anemia, amounting to 5.7% of 227 patients, and similar to that found in the present series. It has been suggested that autoimmune phenomena in CLL resulted from the uncontrolled function of the abnormal lymphocyte clones proliferating in this disease [4]. This suggestion cannot account for the marked difference in the incidence of hemolytic anemia in CLL in the various series published.

The occurrence of one or more additional tumors in CLL patients has been reported to range between 2.5 and 34% [11, 13, 26, 28]. The most frequent solid tumors identified involved the skin, colon and breast. The highly increased incidence reported by HYMAN [13] could be related either to the care with which the patients were followed, or to the more aggressive therapy protocols used, or the increased longevity because of the treatment. Commonly impairment in the immune surveillance is a unifying etiological factor in explaining second primary neoplasms [4]. In line with the observations summarized above, multiple tumors developed only in patients of East European origin and none of Asian-African origin.

Acknowledgments

We express our appreciation to Dr. B. RAMOT from the Haim Sheba Medical Center to Dr. I. TATARSKY from the Rambam Medical Center to Dr. J. PODGAS from the Bellinson Medical Center and to Dr. A. DEVLAMSKY from the Beer Sheva Medical Center for allowing to review the patients' records and for their cooperation. We also thank the staff of the Hematological Clinics and Medical Archives of the above hospitals for their support.

years. The mean survival was less in patients originating from Asia and Africa compared to those of European descent being 2.8 (range 1-6 years) and 4.1 years (range 1-10) respectively

Discussion

The geographical and racial distribution of CLL has been the subject of several reports [27-32]. A high incidence of CLL was reported in Connecticut USA, in the United Kingdom and in Yugoslavia against the low incidence in Poland and Hungary [9]. CLL was reported to occur frequently in the Jewish population of Brooklyn [16] as well as in other countries [21]. A high incidence of leukemia was also reported in the Jewish population of Israel [6]. However, no data is available concerning the incidence of CLL in this country.

A striking difference was found in the prevalence of CLL between Jews from Europe on the one hand and from Asia and Africa on the other. This difference cannot be explained by the former group seeking medical advice more frequently since the comprehensive health insurance encompasses both groups equally and visits to the outpatient clinics in Israel are among the most frequent in the world. It should also be noted that epidemiological studies among the ethnic groups of Israel have disclosed a similar difference between these groups in the distribution of malignant lymphoma [19], carcinoma of the stomach [29], colorectal cancer [18], gall bladder cancer [12] and brain tumors [5]. While we have no explanation for the difference observed in the prevalence of CLL between the different ethnic groups, one can speculate that the constitutional genetic makeup of these groups was the decisive factor in the distribution of CLL among them. It may be of interest to note here that intestinal lymphoma was found to occur exclusively among Middle Eastern Jews and Arabs and was not observed in individuals of European descent [23]. HL-A typing may be of value in investigating this question. Wide variations in the frequencies of HL-A antigens in various ethnic groups are known [30]. Studies performed in CLL patients showed an increased incidence of HL-A 3, HL-A 12 and W15 antigens [30-31]. However, it should be pointed out that environmental factors could not be totally excluded in the pathogenesis of CLL.

There was a progressive decrease in the serum immunoglobulin levels in the majority of the patients, whether they received treatment or not.

- 21 PANTON, P. N. and VALENTINE, E. Chronic lymphocytic leukemia. *Lancet* **i**, 914-916 (1929).
- 22 PIROFFKY, B. Autoimmunization and the autoimmune hemolytic anemia (Williams & Wilkins, Baltimore 1969).
- 23 RAMOT, B. and HULI, N. Clinical and pathological aspects of intestinal lymphoma and the relation to paraproteins of alpha chain Harefesh **76**, 396-398 (1969).
- 24 SCOTT, R. B. Leukemia. *Lancet* **ii**, 1162-1167 (1957).
- 25 SEAMAN, A. J., KOLZ, R. B., PIROFFKY, B., and OSOONO, E. E. Incidence and treatment of acquired hemolytic anemia complicating chronic lymphatic leukemia. *Proc. 6th Int. Congr. Int. Soc. Hemat.*, **P** 857 (Grune & Stratton, New York 1956).
- 26 STANVAKY, K. M., WATSON, T. A., WHITE, E. D., and MIXER, E. M. Chronic lymphatic leukemia and subsequent cancer in the same patient. *Cancer* **36**, 410-414 (1970).
- 27 STENDER, P. E. Cancer race and geography (Williams & Wilkins, Baltimore 1954).
- 28 SWEET, D. L., GOLDBER, H. M., and ULLMANN, E. J. The clinical features of chronic lymphocytic leukemia. *Clin. Haemat.* **6**, 183-202 (1977).
- 29 TULCHINSKY, D. and MODAN, B. Some epidemiological aspects of cancer of the stomach in Israel. *Cancer* **20**, 1311-1316 (1966).
- 30 VIADUTSU, A. O. and ROSE, R. N. HLA antigens. Association with disease. *Immunogenetics* **1**, 305-328 (1974).
- 31 WALFORD, R. L., ZELLER, E., COMBS, L., and KOBRAN, P. HLA specificities in acute and chronic lymphatic leukemia. *Transplant. Proc.* **3**, 1297-1300 (1971).
- 32 WILLS, R. and LAU, K. S. Incidence of leukemia in Singapore and rarity of chronic lymphatic leukemia in Chinese. *B. med. J.* **i**, 759-763 (1960).

References

- 1 BARTIL, M. Alteration du sang remarquable par la prédominance des globules blancs ou muqueux hypertrophie considérable de la rate. *Bull. Soc. med. Hôp. Paris* 3 39 (1856)
- 2 BENTWICH Z. and KUNKEL, H. G. Specific properties of human B and T lymphocytes and alterations in disease. *Transplant Rev* 16: 29-50 (1973).
- 3 BLANCHMAN M. A. DACHE, J. V., HOBBS, J. R. PETIT J. E., and WOLLIDOR, S. M. Immunoglobulins in warm type autoimmune hemolytic anemia. *Lancet* II. 340-344 (1969).
- 4 BURNET F. M. Immunological aspects of malignancy *Lancet* I. 1171-1173 (1967)
- 5 COHEN A. and MODAN, B. Some epidemiological aspects of neoplastic diseases in Israeli migrant population. III. Brain tumors. *Cancer* 22 1323-1328 (1968)
- 6 CROWDY E. V.. *Cancer in man. Etiology and prevention of* (Appleton Century Crofts, New York 1968).
- 7 DAMESEK, W. and GUNZ, F.. *Leukemia* 2nd ed. (Grune & Stratton, New York 1964)
- 8 DAMESEK, W. Chronic lymphatic leukemia as an accumulative disease of immunologically incompetent lymphocytes. *Blood* 29 566-584 (1967)
- 9 DOLL, R., MUIR, C., and WATERHOUSE, J. *Cancer incidence in five continents*, vol. 2 (UICC Publications, 1970).
- 10 FRAUMENI J. F. VOGEL, C. L., and DEVITA, V. T.. Familial chronic lymphocytic leukemia. *Ann. intern. Med.* 71 279-284 (1969).
- 11 GUNZ, F. W. and ANGUS, H. B. Leukemia and cancer in the same patient. *Cancer* 18 145-152 (1965)
- 12 HART J. and MODAN, B.. Cancer of the gall bladder in Israel. *Harefuah* 80-139-140 (1971).
- 13 HYMAN G. A. Increased incidence of neoplasia in association with chronic lymphocytic leukemia. *Scand. J. Haemat.* 6 99-104 (1969).
- 14 Israel Government Year Books (1960-1970)
- 15 LEONCINI, D., FRONTI, A., KORNHOLD, L., and MILLER, D. B. A comparison of paper electrophoretic and immunoelectrophoretic studies of the serum proteins of patients with lymphoma and leukemia. *Oncology* 22. 81-117 (1968)
- 16 MACMAHON B. and KOLLER, E. K. Ethnic differences in the incidence of leukemia. *Blood* 12 1-10 (1957)
- 17 MANCINI, G., CARBONARA, A. O., and HERMANS, J. P. Immunochemical quantitation of antigens by single radial immunodiffusion. *Immunochemistry* 2 235-254 (1965)
- 18 MASS, N. and MODAN, B. Epidemiological aspects of neoplastic disorders in Israeli migrant population. IV. Cancer of the colon and rectum. *J. natn. Cancer Inst.* 42. 529-536 (1969)
- 19 MODAN, B. GOLDMAN B., and SHANI M. Epidemiology of malignant lymphoma in Israel *Harefuah* 73 125-127 (1967)
- 20 OSOODO E. E.. Antiglobulin positive hemolytic anemia. *Archs intern. Med.* 107 313-323 (1961).

- 21 FANTON, F. N. and VALENTINE, F. Chronic lymphocytic leukemia. *Lancet* *i* 914-916 (1929).
- 22 PROFFER, B. Autoimmunization and the autoimmune hemolytic anemia (W. H. Baas & Wilkins, Baltimore 1969).
- 23 RABOT, B. and HIZLI, N. Clinical and pathological aspects of intestinal lymphoma and the relation to paraproteinemias of alpha chain. *Harefesh* 76, 396-399 (1969).
- 24 SCOTT, R. B. Leukemia. *Lancet* *i* 1162-1167 (1957).
- 25 SRAMAK, A. J., KOLER, R. D., PROFFER, B., and OSOON, E. E. Incidence and treatment of acquired hemolytic anemia complicating chronic lymphatic leukemia. *Proc. 6th Int. Congr. Int. Soc. Hemat.*, p. 837 (Grune & Stratton, New York 1956).
- 26 STARVAKY, K. M., WARSON, T. A., WHITE, E. D. and BILSKY, E. M. Chronic lymphatic leukemia and subsequent cancer in the same patient. *Cancer* 26, 410-414 (1970).
- 27 STEDER, P. E. Cancer: race and geography (Williams & Wilkins, Baltimore 1954).
- 28 SWERT, D. L., GOLDBER, H. M., and ULTMAN, E. J. The clinical features of chronic lymphocytic leukemia. *Clin. Haemat.* 6, 185-202 (1977).
- 29 TULCHINSKY, D. and MORAN, M. Some epidemiological aspects of cancer of the stomach in Israel. *Cancer* 20, 1311-1316 (1966).
- 30 VLADUTU, A. O. and ROSE, R. N. HL-A antigens. Association with disease. *Immunogenetics* 7, 305-323 (1974).
- 31 WALFORD, R. L., ZILLER, E., COMPT, L., and KORDAD, P. HL-A specificities in acute and chronic lymphatic leukemia. *Transplant. Proc.* 9, 1297-1300 (1971).
- 32 WELLS, R. and LAU, K. S. Incidence of leukemia in Singapore and rarity of chronic lymphatic leukemia in Chinese. *Br. med. J.* 1, 759-763 (1960).

Collection of *in vitro* Colony-Forming Units from Dogs by Repeated Continuous Flow Leukaphereses¹

P KOVÁCS, C. BRUCH, E. W. HERBST and T. M. FLIEDNER

Department of Clinical Physiology, University of Ulm, Ulm

Key Words: Leukapheresis, Canine leukocytes, Cell culture, Colony formation

Abstract The presence of *in vitro* colony forming units (CFUc) was demonstrated in leukocyte suspensions collected by continuous flow leukaphereses performed serially in dogs. The CFUc yield of one leukapheresis was usually between 10^4 and 10^6 . Higher yields were obtained in the 2nd to 5th leukaphereses than in the first one in the same dog.

The number of CFUc collected during one leukapheresis was about four times the number of CFUc present in the circulating blood at the beginning of the leukapheresis. After a diminution during leukapheresis, the circulating CFUc pool was restored in 2 or 3 days with an increase in the ratio of CFUc to mononuclear cells. These results indicate that CFUc entered the circulating pool during and after leukapheresis and that the population size regulation of canine-circulating CFUc is different from that of the bulk of the mononuclear leukocytes.

A preclinical model of transplantation of haemopoietic stem cell concentrates prepared from peripheral blood leukocytes is being established in our laboratory [7, 11, 12]. For such a model, information on the number of haemopoietic stem and progenitor cells recoverable from canine circulating blood would be essential. Cells capable of forming colonies of granulocytic and/or mononuclear types *in vitro* (colony forming unit in

¹ Research supported by the 'Fraunhofer Gesellschaft', the European Atomic Energy Community and the Deutsche Forschungsgemeinschaft, BIO No 1170 and executed while Dr Kovács was a Visiting Fellow of the International Atomic Energy Agency.

culture = CFUc) are regarded to be granulocytic-macrophage progenitors and can be estimated by the soft agar method [2, 18, 21]. CFUc are present among human [8, 15, 17, 19] and canine [3, 5, 14] peripheral blood leukocytes and can be collected by leukapheresis [3, 17, 22].

We have studied the possibilities and limitations of collecting CFUc containing leukocyte suspensions from dogs by single and repeated continuous flow leukaphereses. Our findings have demonstrated that CFUc can be collected from the peripheral blood of dogs by repeated continuous flow leukapheresis in numbers exceeding by far those present in the circulating blood at the beginning of the collection procedure. In addition, our results indicate that CFUc enter the circulating pool during and after leukapheresis and the population size control of circulating CFUc differs from that of the bulk of the mononuclear leukocytes.

Materials and Methods

Leukapheresis

In 15 beagles of both sexes, weighing 13.1–17.6 kg (mean: 14.7 kg), arterial venous shunts were implanted between the carotid artery and the jugular vein under Halothane anaesthesia 2 or 3 days before the first leukapheresis. Leukapheresis was performed by means of NCI IBM experimental blood cell separator (type 2990) [4] and repeated at 2 or rarely at 3-day intervals up to six times with dog, under the following conditions. At the beginning of each leukapheresis, 500 ml Plas-magel or Neo-Plasmagel (Braun, Melsungen, FRG) was infused intravenously in order to increase red cell sedimentation. For anticoagulation, 10,000 U of heparin and about 750 ml ACD were used. The leukapheresis was continued for 5 h with centrifuge speed of 850 rpm. Buffy coat cells were collected in plastic bag containing 320 U of heparin in 10 ml ACD while plasma and erythrocytes were recombined and retransfused at rate of 50 ml/min. As the upper layer of the erythrocytes contained many leukocytes, an effective collection of leukocytes could not be obtained without some erythrocytes. The contaminating red cells amounted to about 10% (v/v) of the collected cell suspensions as measured by means of micro-haematocrit centrifuge.

In vitro Culture of Leukocytes

For the estimation of the number of CFUc in blood, leukocytes were separated by dextran sedimentation from heparinized blood, washed twice and resuspended in tissue culture medium (see later). The number of mononuclear and polymorphonuclear leukocytes was determined in Neubauer chamber at 500 \times magnification. Leukocytes collected by leukapheresis were washed and differential counts were performed as in the case of leukocytes separated by dextran sedimentation.

Eagle's MEM for spinner cultures (Gibco) was supplemented with 20% horse serum (Flow Labs) as well as with vitamins, amino acids and sodium pyruvate as

cording to PIKE and ROBINSON [20] Cells suspended in the above medium were kept at 37 °C, and 3% agar maintained at 50 °C, was added to a final concentration of 0.3%. The addition of 50 °C agar to the medium already containing the cells did not impair colony growth since in preliminary experiments identical colony numbers were found irrespective of the sequence of the addition of agar and cells to the medium. 1-ml portions of this mixture were plated into 35-mm plastic Petri dishes with 0.2 ml of serum taken from dogs 10 days after 1,200 R whole body X-irradiation. This volume of serum contained an optimal amount of colony-stimulating activity. Although the two batches of dog sera (each pooled from several dogs) used in the present experiments exhibited the same colony stimulating activity all determinations of CFUc from individual dogs were carried out with the same batch of sera.

Cultures were set up at least in triplicate and were incubated at 37 °C in humidified air with 3% CO₂ according to the method of FURKER [9], for 7 days. Colonies, defined as groups of at least 15 cells [16], were counted under a dissecting microscope (magnification 30 ×). For morphological classification, colonies were picked up with a modified Pasteur pipette vitally stained with acridine orange and examined with a fluorescent microscope.

Statistical Analysis of Data

Technical factors sometimes limited the number of leukaphereses performed per dog so that the number of repeated leukaphereses was not uniform. Values given under Results were calculated from all the available data of experiments. For statistical analysis, however data of subsequent leukaphereses were compared only to that of the first leukapheresis of the same dog by means of the t test for paired comparisons [1], after logarithmic transformation. The use of this method was justified because the distribution of the differences between the logarithms of the data of the first and repeated leukaphereses did not differ significantly from a normal distribution, as judged by the Kolmogorov-Smirnov test [10].

Calculations

The number of CFUc/ml blood was calculated on the assumption that the CFUc/mononuclear cell ratio in the leukocytes isolated by dextran sedimentation was equal to that in the original blood sample. The following formulas were used

$$\text{CFUc/ml blood} = C \times M_b/M_p$$

$$\text{CFUc yield from a leukapheresis} = C \times M_b/M_p$$

where C = number of colonies per plate; M_b = number of mononuclear cells in 1 ml blood, M_p = number of mononuclear cells per plate; M_c = number of mononuclear cells collected by the leukapheresis.

CFUc in the total circulating blood = CFUc/ml blood × body weight (kg) × 80 assuming a blood volume of 80 ml/kg of body weight [13].

The efficiency of CFUc removal was calculated by dividing the number of CFUc collected by the number of CFUc that entered the cell separator. As the exact number of CFUc entering the separator was not known, this quantity was estimated by multiplying the mean of the CFUc count/ml blood before and after leukapheresis by the volume of blood that passed through the separator.

Results

In total, 51 leukaphereses were performed in 15 dogs. From all the resulting cell suspensions cultures were set up and showed colony formation. The colony size was between 15 and 400 cells. Of the 7 day-old colonies examined, the percentage of granulocytic, mononuclear and mixed colonies was found to be 75.4 and 19 respectively in cultures of leukapheresis leukocytes ($100\% = 71$) and 86, 6 and 8, respectively in cultures of leukocytes isolated from peripheral blood by dextran sedimentation ($100\% = 358$).

Since the relationship of the number of colonies to that of the mononuclear cells plated was found to be linear between 1 and 10×10^6 mononuclear cells per plate, 5×10^6 mononuclear cells were plated routinely in each dish. In the course of the present experiments, however it was observed that such a linearity existed only when the number of contaminating polymorphonuclear cells did not exceed $25\text{--}30 \times 10^3$ per culture dish. Above this polymorphonuclear concentration an impairment of colony formation occurred with little, if any growth with 40×10^6 polymorphonuclear cells, as reported in detail elsewhere [14]. For this reason, 10

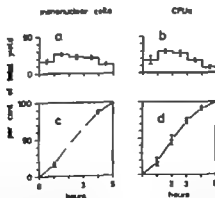


Fig 1 Hourly yields of mononuclear cell (a) and CFUc (b), as well as cumulative collections of mononuclear cells (c) and CFUc (d), expressed as percentage of the total yield of the 5-hour leukapheresis, are plotted against time. Mean values of five leukaphereses; vertical bars represent SE. The CFUc yield of the 5th hour was significantly lower than that of the 2nd, 3rd and 4th hours ($p < 0.001$, $p < 0.005$ and $p < 0.025$, respectively)

Table 1 Yield of repeated leukaphereses

Serial No of leuka phereses	n ¹	× 10 ⁸ polymorpho- nuclear cells		× 10 ⁸ mononuclear cells		CFUc/10 ⁶ mononuclear cells			× 10 ⁶ CFUc		
		median	mean (SE)	median	mean (SE)	median	mean (SE)	> p ²	median (range)	mean (SE)	> p ²
1	15	14.9	14.7 (1.3)	7.8	9.1 (1.2)	11.0	15.3 (8.6)	-	0.8 (0.05-15.2)	2.6 (1.1)	-
2	14	17.8	18.2 (2.5)	7.9	8.7 (1.2)	46.3	80.9 (25.9)	0.001	4.2 (0.8-15.7)	5.8 (1.3)	0.001
3	10	18.5	20.4 (2.7)	8.4	8.6 (0.9)	82.3	83.4 (16.3)	0.001	7.0 (1.4-4.5)	7.6 (2.1)	0.01
4	6	20.6	24.0 (3.7)	6.1	10.6 (2.8)	124	114 (22)	0.01	9.6 (5.2-11.7)	9.4 (1.1)	0.02
5	4	24.5	25.5 (9.6)	5.6	5.5 (0.6)	106	1.6 (47)	0.1	6.6 (2.0-10.3)	6.4 (1.9)	0.3

¹ n = Number of experiments.

² For statistical analysis, the logarithms of data of subsequent leukaphereses were compared to that of the first leukapheresis of the same dog by the *t* test for paired comparisons [1].

out of 24 cultures of leukocytes obtained from the peripheral blood after leukapheresis had to be excluded from further presentation. In most cases, these omitted cultures showed no colony formation.

The number of CFUc collected in one leukapheresis was, with a few exceptions, between 10¹ and 10⁶ 80% of the values exceeding 10³. The CFUc yields of leukaphereses were compared to the number of CFUc in the circulating blood at the beginning of each of 34 leukaphereses and an average of 4 (median 3) circulating blood volumes were found to be depleted of CFUc during a 5 hour cell separation. In these 34 leukaphereses, 12.1 ± 0.4 (mean ± SE) blood volumes were passed through the separator. The efficiency of the CFUc removal was estimated to be 51.7 ± 9.1% (mean ± SE) in the 14 leukaphereses for which the relevant data were available.

In order to learn whether the yield of CFUc was constant during a 5 hour leukapheresis, leukocytes were collected hourly and examined with respect to CFUc content (fig. 1). The yield of mononuclear cells and CFUc was highest during the second hour of leukapheresis, amounting to 27 and 30% of the totals, respectively. Then it decreased gradually so that

Table II CFUc and mononuclear cell content of blood at the beginning of leukaphereses repeated in intervals of 2 or 3 days

Serial No. of leukaphereses		10 ⁶ mononuclear cells/ml blood	CFUc/10 ⁶ mononuclear cells		CFUc/ml blood	
		mean \pm SE	mean \pm SE	> p ^a	mean \pm SE	> p ^a
1	13	2.93 \pm 0.28	23.4 \pm 6.2		71.5 \pm 24.9	
2	11	2.53 \pm 0.25	53.1 \pm 7.7	0.01	133 \pm 23	0.025
3	8	2.54 \pm 0.25	102 \pm 43	0.02	209 \pm 78	0.05
4	4	2.20 \pm 0.41	125 \pm 63	0.05	236 \pm 91	0.05
5	2	2.23 \pm 0.65	64.7 \pm 34.7	0.5	168 \pm 120	0.11

^a = Number of experiments.

For statistical analysis, the logarithms of data of subsequent leukaphereses were compared to that of the first leukapheresis of the same dog by the *t* test for paired comparisons (1).

only 13% of the total mononuclear yield and 8% of the total CFUc yield were collected in the fifth (final) hour of the leukapheresis.

To investigate the possibility that the blood CFUc pool might be exhausted by leukapheresis, the CFUc content of blood was estimated immediately before and after each of 14 leukaphereses. It was found that the number of CFUc/ml of blood decreased during leukapheresis from a mean of 151 to 62 ($p < 0.005$ as calculated by the paired sample *t* test [1]) but never reached zero.

In the presentation of the results of repeated leukaphereses only the first five leukaphereses are included, because a sixth leukapheresis was performed only in 2 dogs. The mononuclear and polymorphonuclear cell yields of repeated leukaphereses were similar to those of the first ones. The CFUc to mononuclear cell ratio and the total yield of CFUc, however, were significantly higher in the second to fourth than in the first leukaphereses (table I).

To observe changes in the circulating CFUc pool during a series of leukaphereses separated by 2 or 3-day intervals, the CFUc content of the blood was determined at the beginning of each leukapheresis. It was the lowest before the first leukapheresis, and was at a higher level before the second to fourth leukaphereses. The number of mononuclear cells in the blood did not change significantly whereas the ratio of CFUc to mononuclear cells increased in the course of repeated leukaphereses (table II).

Table 1 Yield of repeated leukaphereses

Serial No. of leuka phereses	n ¹	× 10 ³ polymorpho- nuclear cells		× 10 ³ mononuclear cells		CFUc/10 ⁶ mononuclear cells			× 10 ³ CFUc		
		median	mean (SE)	median	mean (SE)	median	mean (SE)	> p ²	median (range)	mean (SE)	> p ²
1	15	14.9	14.7 (1.3)	7.8	9.1 (1.2)	11.0	25.3 (8.6)	-	0.8 (0.05-15.2)	2.6 (1.1)	-
2	14	17.8	18.2 (2.5)	7.9	8.7 (1.2)	46.3	80.9 (25.9)	0.001	4.2 (0.8-15.7)	5.8 (1.3)	0.001
3	10	18.5	20.4 (2.7)	8.4	8.6 (0.9)	82.3	83.4 (16.3)	0.001	7.0 (1.4-24.5)	7.6 (2.1)	0.01
4	6	20.6	24.0 (3.7)	6.1	10.6 (2.8)	124	114 (22)	0.0	9.6 (5.2-14.7)	9.4 (1.1)	0.02
5	4	24.5	25.5 (9.6)	5.6	5.5 (0.6)	106	176 (47)	0.1	6.6 (2.0-10.3)	6.4 (1.9)	0.3

¹ n = Number of experiments.

² For statistical analysis, the logarithms of data of subsequent leukaphereses were compared to that of the first leukapheresis of the same dog by the *t* test for paired comparisons [1].

out of 24 cultures of leukocytes obtained from the peripheral blood after leukapheresis had to be excluded from further presentation. In most cases, these omitted cultures showed no colony formation.

The number of CFUc collected in one leukapheresis was, with a few exceptions, between 10⁴ and 10⁶. 80% of the values exceeding 10⁵. The CFUc yields of leukaphereses were compared to the number of CFUc in the circulating blood at the beginning of each of 34 leukaphereses and an average of 4 (median, 3) circulating blood volumes were found to be depleted of CFUc during a 5-hour cell separation. In these 34 leukaphereses, 12.1 ± 0.4 (mean ± SE) blood volumes were passed through the separator. The efficiency of the CFUc removal was estimated to be 51.7 ± 3.1% (mean ± SE) in the 14 leukaphereses for which the relevant data were available.

In order to learn whether the yield of CFUc was constant during a 5 hour leukapheresis leukocytes were collected hourly and examined with respect to CFUc content (fig. 1). The yield of mononuclear cells and CFUc was highest during the second hour of leukapheresis, amounting to 27 and 30% of the totals, respectively. Then it decreased gradually so that

Table II. CFUc and mononuclear cell content of blood at the beginning of leukaphereses repeated in intervals of 2 or 3 days

Serial No. of leukapheresis	n	10 ⁶ mononuclear cells/ml Blood	CFUc/10 ⁶ mononuclear cells		CFUc/ml Blood	
		mean \pm SE	mean \pm SE	> p	mean \pm SE	> p
1	13	2.93 \pm 0.28	23.4 \pm 6.2		71.5 \pm 4.9	
2	11	2.53 \pm 0.25	53.1 \pm 7.7	0.01	133 \pm 4.3	0.05
3	8	2.54 \pm 0.25	102 \pm 45	0.02	209 \pm 78	0.05
4	4	2.20 \pm 0.41	125 \pm 63	0.05	236 \pm 95	0.05
5		2.23 \pm 0.65	64.7 \pm 34.7	0.5	168 \pm 120	0.05

n = Number of experiments.

For statistical analysis, the logarithms of data of subsequent leukaphereses were compared to that of the first leukapheresis of the same dog by the *t* test for paired comparisons (1)

only 13% of the total mononuclear yield and 8% of the total CFUc yield were collected in the fifth (final) hour of the leukapheresis.

To investigate the possibility that the blood CFUc pool might be exhausted by leukapheresis, the CFUc content of blood was estimated immediately before and after each of 14 leukaphereses. It was found that the number of CFUc/ml of blood decreased during leukapheresis from a mean of 151 to 62 ($p < 0.005$ as calculated by the paired sample *t* test [1]) but never reached zero.

In the presentation of the results of repeated leukaphereses only the first five leukaphereses are included, because a sixth leukapheresis was performed only in 2 dogs. The mononuclear and polymorphonuclear cell yields of repeated leukaphereses were similar to those of the first ones. The CFUc to mononuclear cell ratio and the total yield of CFUc, however were significantly higher in the second to fourth than in the first leukaphereses (table I)

To observe changes in the circulating CFUc pool during a series of leukaphereses separated by 2 or 3-day intervals, the CFUc content of the blood was determined at the beginning of each leukapheresis. It was the lowest before the first leukapheresis, and was at a higher level before the second to fourth leukaphereses. The number of mononuclear cells in the blood did not change significantly whereas the ratio of CFUc to mononuclear cells increased in the course of repeated leukaphereses (table II).

Discussion

The attempts in the present studies to collect CFUc from canine blood by continuous flow centrifugation were successful since all cultures of leukapheresis leukocytes showed colony formation. The percentages of granulocytic and mononuclear colonies, as well as the colony size, were similar to those found in cultures of leukocytes isolated from the peripheral blood by dextran sedimentation. This suggests that the differentiating capacity of CFUc collected from the blood by leukapheresis was not altered by the procedure.

The CFUc yield from one leukapheresis exceeded by a factor of four the number of CFUc present in the total circulating blood at the beginning of the leukapheresis. This finding could be explained either by a short transit time of CFUc compared to the 5 hour duration of the leukapheresis or by a mobilization of non-circulating CFUc or both. Repeated leukaphereses at 2 or 3-day intervals were highly effective in increasing the number of CFUc collected from a single donor since the CFUc yields from subsequent leukaphereses were higher than those from the first ones (table I). This probably resulted from the observed increase in CFUc in the peripheral blood after the first leukapheresis (table II) as a correlation was found between the CFUc yield and the number of CFUc/ml blood at the beginning of the leukaphereses ($r = 0.82$ $p < 0.001$ data from 34 leukaphereses).

The CFUc/leukocyte ratio was significantly higher in 36 cells suspensions collected during the second through fifth leukaphereses than in 26 blood samples taken from untreated dogs (29.9 ± 5.4 and 14.9 ± 1.7 CFUc/ 10^4 leukocytes, respectively; mean \pm SE $p < 0.05$). The higher concentration of CFUc in leukapheresis leukocytes may be due, at least in part, to a higher collection efficiency of leukapheresis for CFUc than for the bulk of the other leukocytes, as suggested by MCCREDIE *et al* [17].

It can be established that repeated leukapheresis performed by means of an NCI IBM continuous flow blood cell separator is an effective method of collecting large numbers of CFUc from canine peripheral blood. The high number and concentration of CFUc in leukapheresis leukocytes make them probably advantageous for further cell separation to concentrate canine-circulating CFUc.

During a leukapheresis there was no linear increase with time in the number of CFUc collected (fig. 1). The relatively low yield during the first hour can be explained on technical grounds: it took about 20 min to

produce a buffy coat in the bowl of the cell separator before leukocytes could be collected. From this time on, the collection procedure continued without any technical alterations so, the gradual decrease in the CFUc yield after the second hour of the leukapheresis indicated a progressive diminution of the circulating CFUc pool. This was proven by the observed reduction in the number of CFUc/ml blood. The low CFUc content in the peripheral blood at the end of the leukapheresis could be due to an exhaustion of the presumably extravascular CFUc pool supplying the circulating pool and/or to a limit of CFUc mobilization.

As mentioned earlier the number of CFUc removed from the circulation during a leukapheresis exceeded by a factor of four the number of CFUc present at the beginning of the leukapheresis. The circulating CFUc pool, however was only partially exhausted during such a leukapheresis and was restored, with an overshoot, in 2-3 days (table II). Since proliferation of CFUc within the circulating pool cannot account for the above observations, these data clearly indicate that CFUc entered the circulating pool during and after leukapheresis.

We assume that most, if not all, of the CFUc entering the circulating pool originate from extravascular sites, most likely from the bone marrow. Unfortunately this assumption cannot be tested experimentally. The number of CFUc/ 10^5 nucleated cells in 23 bone marrow specimens aspirated from normal dogs was 116 ± 14 (mean \pm SE). As the total bone marrow cellularity of dogs amounts at least to 10^{10} /kg body weight [6] the number of CFUc removed from a dog in five subsequent leukaphereses (table I) was presumably less than 2% of the CFUc content of the total bone marrow. This is in agreement with our finding that, in the 2 dogs examined in this respect immediately after the fifth leukapheresis, the number of CFUc/ 10^5 nucleated bone marrow cells was well within the normal range.

It cannot be answered as yet whether the increase in circulating CFUc after the first leukapheresis is due to direct feedback mechanisms which cause a mobilization of CFUc from extravascular sites or is influenced indirectly by the removal of large amounts of granulocytes. Other factors, such as infection or the drugs used, should also be taken into consideration. An infection or inflammation at the shunt or other sites are unlikely to have occurred in the course of the present experiments because neither local nor systemic signs of such phenomena were found. Of the drugs applied, Plasmagel, in preliminary experiments, exerted no effect on the number of circulating CFUc, whereas a CFUc-mobilizing effect of the other drugs used (e.g. heparin) remains to be excluded.

The CFUc/mononuclear cell ratio increased in the blood of dogs having undergone repeated leukaphereses (table II) whereas the number of mononuclear cells/ml blood remained unchanged. This indicates that the number of CFUc in blood may change without any parallel alterations in the mononuclear cell count and suggests that canine-circulating CFUc have their own population size control separate from that of the bulk of the mononuclear leukocytes.

Acknowledgements

We are indebted to Dr. H. P. SCHNAPPAUF for the shunt operations, and to Dr. H. D. FLAD and Dr. R. HUGET for their help in leukaphereses, as well as to Dr. B. NELSON (Oak Ridge Associated Universities) for his comments in the course of the preparation of the manuscript, and to Dr. SZ. ROCHLITZ (Kossuth Lajos University Debrecen, Hungary) for his help in statistical analysis. The technical assistance of Miss E. RÜBER, Mrs. A. SAMBOL, Miss B. STENGEL and Mr. S. LIEB is gratefully acknowledged.

References

1. Armitage, P. Statistical methods in medical research, pp. 116-118 (Blackwell, Oxford 1971).
2. BRADLEY T. R. and METCALF D. The growth of mouse bone marrow cells *in vitro*. *Aust. J. exp. Biol. med. Sci.* 44: 287-300 (1966).
3. BRUCH C., HERBST E., W. CALVO, W., HUGET R., FLAD, H. D. and FLIEDNER, T. M. Freezing of blood leukocytes for transplantation into lethally irradiated dogs. In WEDER, OLDHAM and SCHWARZENBERG. La cryoconservation des cellules normales et néoplasiques, pp. 51-62 (Inserm Librairie Ophrys, Paris 1973).
4. BUCKNER, D., EISEL, R., and PERRY S. Blood cell separation in the dog by continuous flow centrifugation. *Blood* 31: 653-672 (1968).
5. DEKILAK FEHR, A. M., CATCHATOURIAN R., and EPSTEIN R. B. Hemopoietic colony forming units in fresh and cryopreserved peripheral blood cells of canines and man. *Exptl. Hematol., Copenh.* 3: 109-116 (1975).
6. DEUMELINCK, K. A., DANCEY J. T., HARKER, L. A., CHENEY B., and FROST, C. A. Marrow erythroid and neutrophil cellularity in the dog. *J. clin. Invest.* 55: 825-832 (1975).
7. CALVO, W., FLIEDNER, T. M., HERBST E., HÖGL, E., and BRUCH, C. Regeneration of blood forming organs after autologous leukocyte transfusion in lethally irradiated dogs. II. Distribution and cellularity of marrow in irradiated and transfused animals. *Blood* 47: 593-601 (1976).
8. CIERVENICK, H. A. and BOGGS, D. R. *In vitro* growth of granulocyte and mononuclear cell colonies from blood of normal individuals. *Blood* 37: 131-135 (1971).

- 9 FULKEIT H. A very simple trick to produce controlled CO₂ concentrations in the gas phase overlying cell cultures. *Experientia* 25: 671-672 (1969).
- 10 FISZ, M. Wahrscheinlichkeitsrechnung und mathematische Statistik (VEB Deutscher Verlag der Wissenschaften, Berlin 1967).
- 11 FLIDNER, T. M., HOLT, E. H., FLAD, H. D., NOTHDEGGER, W. H., CALVO, W., HUOLT, R., ROSE, W. M., SCHNAPPATZ, H. P. and STERNBACH, L. Collection and use of blood stem cells for the treatment of bone marrow aplasia. canine model. In *GOLDMAN and LOWENTHAL. Leukocyte: separation, collection and transfusion*, pp. 771-773 (Academic Press, New York 1975).
- 12 HERBST, E. W., FLIDNER, T. M., CALVO, W., SCHNAPPATZ, H. P. and MITTER, H. Untersuchungen über die Gewinnung hämopoetischer Stammzellen aus dem peripheren Blut von Hunden und über ihre Fähigkeit, die Blutreifebildung zu regenerieren. *Blut* 30: 265-276 (1975).
- 13 HUGHES, R. A., SMITH, E. L., DEVEREAUX, S., and OVERTON, R. C. Changes in cell and plasma volumes in the dog produced by hemorrhage and reinfusion. *Am. J. Physiol.* 189: 49-55 (1957).
- 14 KOVÁCS, P., BRUCE, C., and FLIDNER, T. M. Colony formation by canine hemopoietic cell *in vivo* inhibition by polymorphonuclear leukocytes. *Acta haemat.* 56: 107-115 (1976).
- 15 KUTYCH, J. E. and ROBINSON, W. A. Colony growth of human peripheral white blood cells *in vitro*. *Blood* 37: 136-141 (1971).
- 16 MARSH, J. C., LEVITT, M. and KATZOWITZ, A. The growth of leukocyte colonies *in vitro* from dog bone marrow. *J. Lab. clin. Med.* 79: 1041-1050 (1972).
- 17 MCCREDIE, K. B., HERBST, E. M., and FREEMAN, E. J. Cells capable of colony formation in the peripheral blood of men. *Science* 171: 293-294 (1971).
- 18 METCALF, D. and MOORE, M. A. S. Haemopoietic cells (North-Holland, Amsterdam 1971).
- 19 PARAN, M., SACHS, L., BARAK, Y. and RUMATSKY, E. *In vitro* induction of granulocyte differentiation in hemopoietic cells from leukemic and non-leukemic patients. *Proc. natn. Acad. Sci. USA* 67: 1543-1549 (1970).
- 20 PIER, E. L. and ROBINSON, W. A. Human bone marrow colony growth in agar gel. *J. cell. Physiol.* 76: 77-84 (1970).
- 21 PLUDNIK, D. H. and SACHS, L. The cloning of normal 'mixed' cells in tissue culture. *J. cell. comp. Physiol.* 66: 319-324 (1965).
- 22 WEINER, R. S., RACHMAN, C. M. and YANKEE, R. A. Semicontinuous flow centrifugation for the phenotyping of immunocompetent cells and stem cells. *Blood* 49: 391-397 (1977).

Inorganic Iron Absorption in Subjects with Iron Deficiency Anemia, Achylia gastrica and Alcoholic Cirrhosis Using a Whole-Body Counter¹

A. CELADA, H. RUDOLF V. HERREROS and A. DONATH

Division of Nuclear Medicine, Department of Internal Medicine,
Cantonal Hospital University of Geneva, Geneva

Key Words. Iron absorption · Iron deficiency anemia · Achylia gastrica · Alcoholic cirrhosis · Whole-body counter

Abstract A precise, although technically complicated method is described for the estimation of iron absorption. A steel chamber is used to measure the total radioactivity of the patients. A dose of ^{55}Fe is administered orally and the retention of radioactivity is measured on the 14th day this amount being equivalent to the amount absorbed. In our male group, the mean absorption of inorganic iron was $22.5 \pm 8\%$, and in females it was $43.3 \pm 11\%$. In 10 patients with iron deficiency anemia, the mean was $92.2 \pm 6\%$, in 10 with gastric achylia it was $4.6 \pm 4\%$, and in 12 chronic alcoholics a value of $13.9 \pm 7\%$ was found.

Introduction

In industrialized countries, throughout the world a lack of iron is the most frequent nutritional deficiency. Attempts to fortify various foods with iron have so far proved unsuccessful in preventing iron deficiency [19]. Since the demonstration, in 1937 by MC CANCE and WIDDOWSON [21] that the physiologic iron losses of the human organism were small it is generally accepted that the body stores of this metal remain constant by modulating its absorption. Although studied extensively both in humans and animals, the basic control mechanisms of iron absorption are not yet understood [14].

In this study we have used a method for the measurement of iron absorption which is extremely precise, although technically complex. The

¹ Supported by the Swiss Foundation for Scientific Research, No. 3.752.72.

results obtained in normal subjects are presented together with those of subjects with iron deficiency anemia, achylia gastrica and chronic alcoholism.

Material and Methods

A control group of 20 healthy volunteers of both sexes, aged 20-36 years, from the staff of the University Cantonal Hospital of Geneva was examined. They did not present any sign of anemia. For ethical reasons, neither bone marrow examination nor liver biopsy was performed. 12 alcoholic male subjects with hepatic cirrhosis and different degrees of hepatic dysfunction were also examined; the diagnoses had been established according to clinical, biological criteria and histological examination of hepatic biopsy. The main criterion of selection of this group was an increase of the deposits of iron in the parenchyma, as shown by Prussian blue coloration of the hepatic tissue. All had been in hospital for more than 1 month, undergoing alcoholic detoxication, and had received multivitaminic preparations including folic acid. There was no evidence of iron deficiency in either group, as proven by normal bone marrow examination, hematocrit, hemoglobin, serum iron and transferrin saturations were also normal.

10 patients who showed iron deficiency following chronic losses of blood in the gastrointestinal or genital tracts were also studied. The diagnostic criteria were those suggested by BARON and FROM [1]: absence of iron in the stroma of the bone marrow, lowering of the hematocrit, hemoglobin, serum iron and transferrin saturation, with an increase of total iron-binding capacity. Finally 10 patients with histamine-resistant achylia gastrica were studied; only 1 of these had iron deficiency and 4 were anemic (hematocrit below 35%). The iron absorption test has been performed when they presented clinical picture of megaloblastic anemia and had not been treated.

The percentage of iron absorption was determined by using whole-body counter. The total body radioactivity is measured after ingestion of test dose of ^{59}Fe and again 14 days later. Under these conditions, the amount of ^{59}Fe retained is equivalent to the amount absorbed, since the absorbed isotope cannot be eliminated.

The whole-body counter consists of steel chamber whose internal measures are $1.9 \times 2.7 \times 2.34\text{ m}$. The walls are 18 cm thick and the chamber weighs approximately 50 tons. In order to obtain the steel with as little intrinsic radioactivity as possible, old railway lines built before 1930 were chosen. The chamber has door 1.98 m high and 0.80 m wide. All of these measures are necessary to obtain the lowest radioactivity level possible, so that minimum doses could be administered in our test.

The detector consists of a single crystal of sodium iodide activated by thallium, cylindrical in shape, with diameter of 203 mm and a height of 102 mm. It is connected to three photomultipliers, 76 mm in diameter. The crystal is situated at 1.8 m from the base of the chamber in the center of the 1.9-meter arc on which the subject lies. Figure 1 is diagram of the chamber with its various fixtures. The room containing the whole-body counter is air-conditioned at temperature of 22 °C. A constant record of temperature and humidity levels is kept in the room.

Inorganic Iron Absorption in Subjects with Iron Deficiency Anemia, Achylia gastrica and Alcoholic Cirrhosis Using a Whole-Body Counter¹

A. CELADA, H. RÜDOLF, V. HERREROS and A. DONATH

Division of Nuclear Medicine, Department of Internal Medicine
Cantonal Hospital University of Geneva, Geneva

Key Words. Iron absorption · Iron deficiency anemia · Achylia gastrica · Alcoholic cirrhosis · Whole-body counter

Abstract A precise, although technically complicated method is described for the estimation of iron absorption. A steel chamber is used to measure the total radioactivity of the patients. A dose of ^{59}Fe is administered orally and the retention of radioactivity is measured on the 14th day this amount being equivalent to the amount absorbed. In our male group, the mean absorption of inorganic iron was $22.5 \pm 8\%$, and in females it was $43.3 \pm 11\%$. In 10 patients with iron deficiency anemia, the mean was $92.2 \pm 6\%$, in 10 with gastric achylia it was $4.6 \pm 4\%$, and in 12 chronic alcoholics a value of $13.9 \pm 7\%$ was found.

Introduction

In industrialized countries, throughout the world a lack of iron is the most frequent nutritional deficiency. Attempts to fortify various foods with iron have so far proved unsuccessful in preventing iron deficiency [19]. Since the demonstration, in 1937 by McCANCE and WIDDOWSON [21] that the physiologic iron losses of the human organism were small, it is generally accepted that the body stores of this metal remain constant by modulating its absorption. Although studied extensively both in humans and animals, the basic control mechanisms of iron absorption are not yet understood [14].

In this study we have used a method for the measurement of iron absorption which is extremely precise, although technically complex. The

Supported by the Swiss Foundation for Scientific Research, No. 3.752/72.

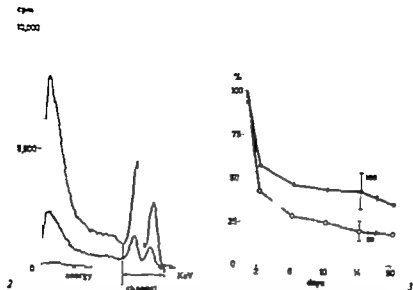


Fig 2 Spectra which record in the measurement of iron absorption with whole-body counter. The top line equals 100% radioactivity after ingestion of the dose, the bottom line is the basic radioactivity and the middle line represents remaining activity after 14 days, which equals the amount absorbed.

Fig 3 Retention of ^{59}Fe after a test dose administered in 10 male volunteers (○) and 10 female volunteers (●). On the 14th day the difference between males and females is significant ($p < 0.001$). SD = Standard deviation.

by the International Committee [17] was used for the serum iron and that of RAMSAL [26] for the total iron-binding capacity.

Student's *t* test was used to evaluate the results.

Results

In the volunteer group, the retention of radioactive iron was measured on the 4th, 7th, 11th, 14th and 90th days after administering the test dose. The results and those of the hematocrit, serum iron and transferrin saturation are given in tables I and II. In figure 3 it can be seen that the iron retention progressively diminished in all cases. At the end of the 14th day when the retention of iron equalled its absorption, there was a difference between the female and male groups ($p < 0.001$). After 90 days, the retention in the female group was higher than in the male group. The average difference between the retentions on the 14th and 90th days was

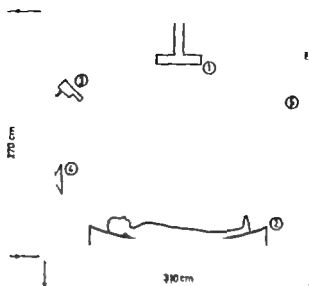


Fig 1 Diagram of the whole-body counter 1 = Crystal 2 = arc on which patients are measured 3 = closed-circuit television 4 = Intercom 5 = door

The spectrum of radioactivity are registered with a completely transistorized Didac 800 impulse analyzer (Intertechnique, France). The cumulative values on a magnetic tape can be subsequently registered numerically on an IBM typewriter (model B) on an Addo X calculating machine or else graphically (Brow strip chart recorder RCL, model 31014)

All measurements are made during a 10-min period to give a sufficient number of counts allowing statistical evaluation of the results. The readings are taken after overnight fasting, to determine the initial background radioactivity. Immediately afterwards, the isotope is administered orally. In our studies, we have chosen the test dose proposed by HENAUSS [16] which consists in 10 moles (= 0.558 mg) of ^{59}Fe (approximately $1 \mu\text{Ci}$ activity: Institut fédéral de Recherches en matière de réacteurs, Würenlingen, Switzerland) + 100 moles (= 17.6 mg) L-ascorbate in 0.001 N HCl in 100 ml of water

To ensure a relatively generalized distribution of isotope throughout the body the total radioactivity was measured 4 h after the isotope [^{59}Fe] was administered. For the measurements with ^{59}Fe , the two energy peaks (1 098 and 1,297 keV) were added. Figure 2 shows the three spectrums which correspond to a routine measurement. The following formula is used to calculate the absorption of iron

$$\text{Absorption (\%)} = \frac{\text{cpm day 14} - \text{background}}{\text{cpm day 1} - \text{background}} \times 100 \times F$$

where cpm means counts per minute and F is the correction factor for the radioactivity decay of ^{59}Fe .

Hematological examinations were carried out by standard methods [6]. The erythrocyte and leukocyte counts and also the hematocrit, hemoglobin and globular indices were made with a laser ray counter (Hemac 630 L). The method proposed

Table III Iron absorption in 10 patients with iron deficiency anemia

Age years	Sex	Hematocrit	Serum iron $\mu\text{g}/100\text{ ml}$	Transferrin saturation	Iron absorption %
35	F	21	40	15	83.4
52	F	25	35	9	95.1
45	F	15	20	10	100.0
48	F	12	35	5	87.2
73	F	25	15	1	99.0
68	F	26	90	18	92.1
56	F	20	85	11	83.6
67	F	17	30	7	86.3
52	M	28	45	12	90.5
64	M	15	35	15	100.0
\bar{x}					92.20
SD					6.09

Table IV Iron absorption in 10 patients with achylia gastrica

Age years	Sex	Hematocrit %	Serum iron $\mu\text{g}/100\text{ ml}$	Transferrin saturation %	Iron absorption %
65	F	40	105	31	3.1
73	F	32	85	25	8.0
88	F	30	95	29	0.5
78	F	33	110	35	0.3
79	F	28	80	24	1.2
75	F	15	30	17	10.7
72	M	45	110	33	6.2
69	M	38	140	30	4.0
81	M	42	90	32	1.5
79	M	46	115	37	10.7
\bar{x}					4.62
SD					4.48

male controls. The absorption in the patients with iron deficiency anemias was higher than that of the controls ($p < 0.001$). In the group of patients with achylia gastrica, the iron absorption was significantly lower than in the controls ($p < 0.001$).

Table V shows the results of the absorption of iron in 12 chronic alcoholics who had abstained from alcohol for over 1 month in hospital. The

Table I Iron retention in 10 normal males on successive days

Age years	Hematocrit %	Serum Iron µg/100 ml	Transferrin saturation %	Iron retention, %				
				day 4	day 7	day 11	day 14	day 90
30	45	120	30	41.4	37.9	33.7	31.4	28.0
25	48	105	32	29.0	29.0	27.0	27.0	20.0
34	44	80	29	55.3	37.5	30.8	28.9	26.8
25	47	110	31	79.1	40.1	27.5	16.4	13.2
22	46	140	32	10.1	6.2	5.5	5.4	4.7
27	43	120	34	47.3	39.8	36.3	34.2	30.2
36	48	105	30	47.0	23.3	20.1	15.4	11.8
35	45	100	28	42.2	26.1	23.0	20.2	18.1
38	43	115	35	56.6	35.5	28.1	25.0	21.3
24	47	95	29	44.1	29.2	24.2	21.1	19.7
\bar{x}				45.21	30.46	25.61	22.50	19.27
SD							8.63	

Table II Iron retention in 10 females on successive days

Age years	Hematocrit %	Serum Iron µg/100 ml	Transferrin saturation %	Iron retention,				
				day 4	day 7	day 11	day 14	day 90
22	35	90	28	63.2	55.5	55.4	54.2	45.0
26	41	105	33	57.3	39.3	37.3	36.0	33.7
32	38	120	31	89.0	64.2	61.7	58.7	51.4
20	46	110	33	51.4	44.1	43.0	42.0	38.0
27	39	85	30	43	20.2	18.2	18.1	17.0
35	43	100	40	59.0	54.1	52.3	52.6	47.0
28	44	110	29	60.0	52.1	50.1	49.2	44.1
26	37	95	32	62	50.3	45.0	45.0	39.2
23	39	80	35	53.1	40.0	38.2	37.1	30.0
20	42	90	33	51.2	42.0	40.5	40.2	34.2
\bar{x}				56.99	46.37	44.12	43.30	37.95
SD							11.6	

5.35% for the female group and 3.14% for the male group. The difference between the two groups was significant ($p < 0.025$).

The results from the 10 patients with iron deficiency anemia and the 10 with gastric achylia are shown in tables III and IV along with the hematocrit, serum iron and transferrin saturation. In figure 4 the absorption of iron by these two groups of patients is compared with that of the

Table III. Iron absorption in 10 patients with iron deficiency anemia

Age years	Sex	Hematocrit	Serum iron $\mu\text{g}/100\text{ ml}$	Transferrin saturation	Iron absorption %
35	F	21	40	15	88.4
52	F	25	35	9	95.1
65	F	15	20	10	100.0
48	F	12	35	5	87.2
73	F	28	15	12	99.0
68	F	26	50	16	92.1
56	F	20	18	11	83.6
67	F	17	30	7	86.3
52	M	28	45	12	90.5
64	M	15	35	15	100.0
\bar{x}					92.20
SD					6.09

Table IV. Iron absorption in 10 patients with achylia gastrica

Age years	Sex	Hematocrit %	Serum iron $\mu\text{g}/100\text{ ml}$	Transferrin saturation	Iron absorption %
65	F	40	105	31	3.1
73	F	32	85	25	8.0
84	F	30	95	29	0.5
78	F	33	110	35	0.3
79	F	28	80	4	1.2
75	F	15	30	17	10.7
72	M	45	110	33	6.2
69	M	38	140	30	4.0
81	M	42	90	32	1.5
79	M	46	115	37	10.7
\bar{x}					4.62
SD					4.48

male controls. The absorption in the patients with iron deficiency anemias was higher than that of the controls ($p < 0.001$). In the group of patients with achylia gastrica, the iron absorption was significantly lower than in the controls ($p < 0.001$).

Table V shows the results of the absorption of iron in 12 chronic alcoholics who had abstained from alcohol for over 1 month in hospital. The

Table V Iron absorption in 12 chronic alcoholics

Age years	Sex	Hematocrit %	Serum iron $\mu\text{g}/100\text{ ml}$	Transferrin saturation	Iron absorption
62	M	45	90	30	11.2
54	M	47	130	32	12.4
48	M	42	110	29	3.0
59	M	46	120	35	11.8
65	M	48	95	34	22.9
39	M	45	125	29	28.8
55	M	48	115	31	9.6
59	M	39	90	28	14.3
69	M	45	110	33	21.3
72	M	46	1.5	32	12.4
45	M	43	115	35	5.3
62	M	40	130	30	14.5
\bar{x}					13.96
SD					7.30

absorption
(%)

100



75

50

25



0

ACHYLIA
GASTRICANORMAL
MALESIRON
DEFICIENCY \bar{x}

4.60

22.47

92.20

SD

4.48

8.64

8.09

Fig 4 Iron absorption in 10 patients with achylia gastrica and 10 with iron deficiency anemia, compared with 10 male volunteers. The shaded area represents 1 standard deviation (SD) and the black line the average (\bar{x}).

result for this group was slightly lower than in the controls of the same sex, but the difference was not significant ($p > 0.50$).

Discussion

At the present time, two methods are commonly used to measure iron absorption. In one, an oral dose of ^{59}Fe and a parenteral dose of ^{59}Fe are given and the incorporation of ^{59}Fe and ^{55}Fe in the erythrocytes is estimated after 14 days. The absorption can be calculated in relation to the oral doses administered. This method has certain disadvantages. The results are inaccurate if there is a deficiency in the incorporation of iron into the red cells. Moreover the results obtained with this method do not correlate with fecal excretion of radioactive iron [16].

VAN HOEK and CONRAD [28] were the first to use the whole-body counter to measure the absorption of iron, and this method has been used subsequently by others [3 10 12, 13 25]. Whole-body counting, where by the total retention of radioactive iron is estimated, is the most sensitive method [3 16]. A further advantage is that the low doses of radioactivity administered allow a number of measurements to be made with only negligible risk. The main disadvantage of the method is the cost of the apparatus and the need for specialized staff to operate it.

In using the whole-body counter in iron absorption studies it is important that all measurements be made at a time when the distribution of radioactivity is as homogeneous as possible. For background measurements and those after 14 days or later this condition is realized. Immediately after absorption of the test dose, all the radioactivity remains concentrated in the stomach, whereas 4 h later there is a much more uniform distribution. For this reason we measured the 100% radioactivity value at this time [13 27].

Iron absorption is a fairly unstable value which varies from one individual to another and even in the same subject, if the experiment is repeated. A study of iron absorption must therefore include a sufficiently large group of subjects to allow statistical evaluation of the results [9].

In our experiments, we used the same test dose as HENRICH [16]. The amount of inorganic iron he gives presents the advantage of a relatively high iron absorption rate in normal subjects, so that it allows to diagnose as well as malabsorption as an overabsorption. It is, however clear that these figures do not apply to food iron absorption. The results in the con-

Table V Iron absorption in 12 chronic alcoholics

Age years	Sex	Hematocrit %	Serum Iron $\mu\text{g}/100\text{ ml}$	Transferrin saturation %	Iron absorption
62	M	45	90	30	11.2
54	M	47	130	32	12.4
48	M	42	110	29	3.0
59	M	46	120	35	11.8
65	M	48	95	34	22.9
39	M	45	125	29	28.8
55	M	48	115	31	9.6
59	M	39	90	28	14.3
69	M	45	110	33	21.3
72	M	46	125	32	1.4
45	M	43	115	35	5.3
62	M	40	130	30	14.5
\bar{x}					13.96
SD					7.30

absorption
(%)

100



75

50

25



0

ACHYLIA
GASTRICANORMAL
MALESIRON
DEFICIENCY \bar{x}

4.60

22.47

32.20

SD

4.68

8.84

8.09

Fig 4 Iron absorption in 10 patients with achylia gastrica and 10 with iron deficiency anemia, compared with 10 male volunteers. The shaded area represents 1 standard deviation (SD) and the black line the average (\bar{x}).

result for this group was slightly lower than in the controls of the same sex, but the difference was not significant ($p > 0.50$).

Discussion

At the present time, two methods are commonly used to measure iron absorption. In one, an oral dose of ^{55}Fe and a parenteral dose of ^{59}Fe are given and the incorporation of ^{55}Fe and ^{59}Fe in the erythrocytes is estimated after 14 days. The absorption can be calculated in relation to the oral doses administered. This method has certain disadvantages. The results are inaccurate if there is a deficiency in the incorporation of iron into the red cells. Moreover the results obtained with this method do not correlate with fecal excretion of radioactive iron [16].

VAN HOEK and CONRAD [28] were the first to use the whole-body counter to measure the absorption of iron, and this method has been used subsequently by others [3 10 12, 13 25]. Whole-body counting, whereby the total retention of radioactive iron is estimated, is the most sensitive method [3 16]. A further advantage is that the low doses of radioactivity administered allow a number of measurements to be made with only negligible risk. The main disadvantage of the method is the cost of the apparatus and the need for specialized staff to operate it.

In using the whole-body counter in iron absorption studies it is important that all measurements be made at a time when the distribution of radioactivity is as homogeneous as possible. For background measurements and those after 14 days or later this condition is realized. Immediately after absorption of the test dose, all the radioactivity remains concentrated in the stomach, whereas 4 h later there is a much more uniform distribution. For this reason we measured the 100% radioactivity value at this time [13 27].

Iron absorption is a fairly unstable value which varies from one individual to another and even in the same subject, if the experiment is repeated. A study of iron absorption must therefore include a sufficiently large group of subjects to allow statistical evaluation of the results [9].

In our experiments, we used the same test dose as HENRICH [16]. The amount of inorganic iron he gives presents the advantage of a relatively high iron absorption rate in normal subjects, so that it allows to diagnose as well as malabsorption as an overabsorption. It is, however, clear that these figures do not apply to food iron absorption. The results in the con-

trol group of males were in fact similar ($22.5 \pm 8.6\%$) to those of HEINRICH ($24 \pm 10\%$). In the female group our levels ($43.3 \pm 11.6\%$) are higher than in HEINRICH's female control group ($32 \pm 10\%$) probably due to the fact that in our group there were some cases of subclinical iron deficiency. These cases could not be excluded since marrow examination was not done, the only criteria used for their selection being the hematocrit, the serum iron and the transferrin saturation. The fractional measurements of these two groups (fig. 3) demonstrate the two phases in the elimination of the radioactivity. An initial decrease can be observed in the first 4 days after the ingestion of radioactive iron. This decrease corresponds to the part not bound to the intestinal mucosa which is excreted with the stools at an early stage. Between the 4th and 7th days, another decrease is observed which is probably related to the iron fixed to the mucosa but not absorbed which passes to the intestinal lumen with the desquamation of the mucosa and which is eliminated. From the 7th day onwards, the amount of radioactivity diminishes very slowly being related to the obligatory physiological iron loss.

After 90 days, a decrease in the retention of radioactive iron is noted, this was greater in the female than in the male group probably because of menstruation, since young women were used in the experiment.

The level of iron deposits in the organism is one of the main factors known to regulate iron absorption [14]. In the group of patients with iron deficiency anemia, the iron absorption was very high ($92.2 \pm 6\%$) very similar to that of the group tested by HEINRICH [16] ($93 \pm 6\%$).

It is known that hydrochloric acid is essential for the absorption of inorganic iron since in its absence iron precipitates at a pH above 5 [8]. Moreover it is thought that the other components of the gastric juice have an important role, especially a mucopolysaccharide with a molecular weight around 200 000 which possibly binds the iron in foodstuff and which effectively contributes to its absorption [18]. In patients who have undergone gastrectomy and in those with complete achylia gastrica, the absorption of iron is almost zero [22]. Our results in the group of patients with achylia gastrica also show their inability to absorb iron. It is possible that achylia gastrica may be directly responsible for the observed iron deficiency anemia in some cases. We have recently studied 2 patients with severe iron deficiency anemia without any evidence of hemorrhage, but who showed complete achylia gastrica and malabsorption of iron. 1 of them showed no improvement when treated orally with an iron preparation, but when the iron administration was given parenterally his recov-

ery was spectacular. It has also been thought that a deficiency of gastric secretion may be one cause of the iron deficiency anemia observed in children [15].

Many authors have shown, by studying the histology or chemistry of hepatic biopsies, that chronic alcoholics have increased iron deposits [24]. This indicates that iron absorption may be increased in these patients. Two explanations have been suggested for this supposed overabsorption of iron. The first postulates a relation between pancreatic secretions, which are frequently diminished in alcoholics, and iron absorption [5, 11, 20]; however certain experiments have shown that pancreatic juice seems to play no part in the absorption of iron [2, 4, 23]. Secondly it has been suggested that alcohol has a direct influence on iron absorption [7]. In our alcoholic group we found no increase in iron absorption, which was on the contrary slightly diminished. But this group of patients had abstained from alcohol for over 1 month. The increased iron load observed in certain hepatic dysfunctions caused by alcohol may be linked to a direct effect on the intestinal mucosa and on the mechanism which regulate the overall absorption of iron.

References

1. BLITTON, D. F. and FINCH, C. A. The diagnosis of iron deficiency anemia. *Am. J. Med.* 37: 62-70 (1964).
2. BALCERZAK, S. P., PETERGEL, W. W. and HEDGECOCK, E. W. Iron absorption in chronic pancreatitis. *Gastroenterology* 53: 257-264 (1967).
3. BANGORIAN, R. M., O'BRIEN, J. R. D. and WITTE, L. J. Studies in iron metabolism. IV. Iron absorption in experimental iron deficiency. *Blood* 20: 532-546 (1962).
4. BRUCE, J. C. and DAVIS, A. E. The exocrine pancreas and iron absorption. *Austral. Ann. Med.* 15: 36-39 (1966).
5. CALLENDER, S. T. and MALPAS, J. S. Absorption of iron in cirrhosis of liver. *Br. med. J.* 4: 1516-1518 (1963).
6. CARTWRIGHT, G. E. *Diagnostic laboratory hematology* (Grune & Stratton, New York 1968).
7. CHARLTON, R. W., JACOBS, P., SEFTHEL, H. and BOTHWELL, T. H. Effect of alcohol on iron absorption. *Br. med. J.* 1: 1477-1479 (1964).
8. COOK, J. D., BROWN, G. N. and VALERIO, L. S. The effect of achylia gastrica on iron absorption. *J. clin. Invest.* 42: 1185-1191 (1964).
9. COOK, J. D., LAYRINE, M., and FINCH, C. A. The measurement of iron absorption. *Blood* 33: 421-429 (1969).
10. COOK, J. D., PALMER, H. E., PAMTHORP, K. G. and FINCH, C. A. The measurement of iron absorption by whole-body counting. *Physica Med. Biol.* 15: 467-473 (1970).
11. DELLER, D. J. Iron⁵⁹ absorption measurements by whole body-counting, studies

trol group of males were in fact similar ($22.5 \pm 8.6\%$) to those of HEINRICH ($24 \pm 10\%$). In the female group, our levels ($43.3 \pm 11.6\%$) are higher than in HEINRICH's female control group ($32 \pm 10\%$) probably due to the fact that in our group there were some cases of subclinical iron deficiency. These cases could not be excluded since marrow examination was not done, the only criteria used for their selection being the hematocrit, the serum iron and the transferrin saturation. The fractional measurements of these two groups (fig. 3) demonstrate the two phases in the elimination of the radioactivity. An initial decrease can be observed in the first 4 days after the ingestion of radioactive iron. This decrease corresponds to the part not bound to the intestinal mucosa which is excreted with the stools at an early stage. Between the 4th and 7th days, another decrease is observed which is probably related to the iron fixed to the mucosa but not absorbed, which passes to the intestinal lumen with the desquamation of the mucosa and which is eliminated. From the 7th day onwards, the amount of radioactivity diminishes very slowly being related to the obligatory physiological iron loss.

After 90 days, a decrease in the retention of radioactive iron is noted; this was greater in the female than in the male group, probably because of menstruation, since young women were used in the experiment.

The level of iron deposits in the organism is one of the main factors known to regulate iron absorption [14]. In the group of patients with iron deficiency anemia, the iron absorption was very high ($92.2 \pm 6\%$) very similar to that of the group tested by HEINRICH [16] ($93 \pm 6\%$).

It is known that hydrochloric acid is essential for the absorption of inorganic iron, since in its absence iron precipitates at a pH above 5 [8]. Moreover, it is thought that the other components of the gastric juice have an important role, especially a mucopolysaccharide with a molecular weight around 200 000 which possibly binds the iron in foodstuff and which effectively contributes to its absorption [18]. In patients who have undergone gastrectomy and in those with complete achylia gastrica the absorption of iron is almost zero [22]. Our results in the group of patients with achylia gastrica also show their inability to absorb iron. It is possible that achylia gastrica may be directly responsible for the observed iron deficiency anemia in some cases. We have recently studied 2 patients with severe iron deficiency anemia without any evidence of hemorrhage, but who showed complete achylia gastrica and malabsorption of iron. 1 of them showed no improvement when treated orally with an iron preparation but when the iron administration was given parenterally his recov-

Acta haemat. 60: 193-200 (1978)

β -Thalassemia in Sicily

Hematological and Biochemical Studies¹

G. SCHILARDÒ, S. MUSUMECI, G. PIZZARELLI, L. DI GREGORIO, A. FISCHER
and G. RUSSO

Department of Pediatrics, University of Catania, Catania

Key Words: Hemoglobin synthesis β^0 Thalassemia β^+ Thalassemia
Retti-Greppi-Micheli disease

Abstract The degree of imbalance in β^0 Th and β^+ Th as well as the frequency of the two forms in Sicilian β -thalassemic subjects have been studied. The hemoglobin synthesis in Retti-Greppi-Micheli disease (RGMD) and in the β -thalassaemia trait has also been studied. In an unselected thalassaemic population, about 30% have been found to be β^0 Th. Both groups of β^0 Th and β^+ Th showed severe imbalance with $\alpha/\text{non-}\alpha$ ratio of 4.22 ± 1.88 (SD) and 3.46 ± 1.36 , respectively. This difference was not statistically significant. In RGMD the $\alpha/\text{non-}\alpha$ ratio was 2.12 ± 0.36 while in the β -thalassaemia trait it was 1.76 ± 0.35 .

The β -thalassemias are the most common disorders of globin chain synthesis in Sicily [1]. During the last 10 years different groups have shown that in β -thalassemias the molecular defect is total (β^0 Th type) or partial (β^+ Th type) depression of normal β -chain synthesis [2-7].

Unlike Ferrara where only the β^0 Th is present, in Sicily β -thalassaemia is heterogeneous, and both forms are present [8]. The defect of β -chain synthesis causes a relative increase in the α -chain in both forms, so that the $\alpha/\text{non-}\alpha$ ratio which is balanced in normal subjects, is unbalanced in the β -thalassemias. In the β -thalassaemia trait, this ratio is above 1.50; in homozygotes for β -thalassaemia the imbalance is more severe with an $\alpha/\text{non-}\alpha$ ratio above 3 [9].

This work is dedicated to the memory of Prof. P. PARANZO, who first described cardiomegaly in Cooley's disease.

Received: October 3, 1977; accepted: February 15, 1978.

- in alcoholics, cirrhosis, haemochromatosis and pancreatitis. *Am. J. dig. Dis.* 10 249-258 (1965).
- 12 DELWAIDE, P. A. Mesure de l'absorption digestive du Fe^{59} au moyen d'un «anthropogammamètre» à scintillateur organique. *Nouv. Revue fr. Hémat.* 12. 683-689 (1972).
 - 13 DYMCK, I. W., GODFREY III E., and WILLIAMS, R. A comparison of methods for the determination of iron absorption using a whole-body counter. *Physics Med. Biol.* 16 269-273 (1971).
 - 14 FORTH, W. and RUMMEL, J. Iron absorption. *Physiol. Rev.* 53 724-792 (1973).
 - 15 GROSS, S. J., STUART M. J., SWENDER, P. T. and OSKI, F. A. Malabsorption of iron in children with iron deficiency. *J. Pediatr.* 88 795-799 (1976).
 - 16 HEDRICK, H. C. Intestinal iron absorption in man, method of measurements, dose relationship, diagnostic and therapeutic applications. In HALLBERG, H., WERTH, and VANNOTTI. Iron deficiency pathogenesis, clinical aspects and therapy pp. 213-298 (Academic Press, London 1970).
 - 17 International Committee for Standardization in Hematology. Proposed recommendations for measurement of serum iron in human blood. *Br. J. Haematol.* 20 451-453 (1971).
 - 18 JACOBS, A. The mechanism of iron absorption. *Clin. Haematol.* 2 323-337 (1973).
 - 19 LAYRIST, M. Dietary iron absorption. In KIEP. Iron metabolism and its disorders, pp. 25-33 (Excerpta Medica, Amsterdam 1975).
 - 20 LINSCHER, W. G., GREENBERG M. S., MOORE, E. W. and CHALMERS, T. C. Absorption in the proximal small intestine in patients with cirrhosis. *Gastroenterology* 46 662-670 (1964).
 - 21 MAC CANCE, H. A. and WIDOWSON, E. M. Absorption and excretion of iron. *Lancet* i. 680-684 (1937).
 - 22 MAGNUSSON, B. E. O. Iron absorption after antrectomy with gastroduodenostomy. *Scand. J. Haematol., suppl.* 46, pp. 1-111 (1976).
 - 23 MURRAY M. J. and STEIN N. Effect of pancreas extracts on Fe^{59} absorption by anemic rats. *J. Lab. clin. Med.* 70 361-364 (1967).
 - 24 POWELL, L. W. The role of alcoholism in hepatic iron storage disease. *Ann. N. Y. Acad. Sci.* 52 124-134 (1975).
 - 25 PRICE, D. C., COHN S. H., WASSERMAN L. R., REINGSTEIN P. G. and CROW KITT, E. P. The determination of iron absorption and loss by whole-body counting. *Blood* 20 517-530 (1962).
 - 26 RAMSAY V. N. M. The determination of the total binding capacity of serum. *Clinica chim. Acta* 2 221-226 (1957).
 - 27 SCHIFFER, L. M., PRICE, D. C., CUTNER, J., COHN, S. H., and CROWKITT, E. P. A note concerning the 100 per cent value in iron absorption studies by whole body counter. *Blood* 23 757-760 (1964).
 - 28 VAN HOEK, R. and COMRAD M. E. Iron absorption. Measurement of ingested iron⁵⁹ by a human whole-body liquid scintillation counter. *J. clin. Invest.* 40 1153-1159 (1961).

Acta haemat. 60: 193-200 (1978)

β -Thalassemia in Sicily

Hematological and Biochemical Studies

G. SCITILIRÒ, S. MUSUMECI, G. PIZZARELLI, L. DI GREGORIO, A. FISCHER
and G. RUSSO

Department of Pediatrics, University of Catania, Catania

Key Words: Hemoglobin synthesis β^0 Thalassemia β^+ Thalassemia
Rieti-Greppi-Micheli disease

Abstract. The degree of imbalance in β^0 Th and β^+ Th as well as the frequency of the two forms in Sicilian β -thalassemic subjects have been studied. The hemoglobin synthesis in Rieti-Greppi-Micheli disease (RGMD) and in the β -thalassemia trait has also been studied. In an unselected thalassemic population, about 30% have been found to be β^0 Th. Both groups of β^0 Th and β^+ Th showed severe imbalance with $\alpha/\text{non-}\alpha$ ratio of 4.22 ± 1.88 (SD) and 3.46 ± 1.36 , respectively. This difference was not statistically significant. In RGMD the $\alpha/\text{non-}\alpha$ ratio was 2.12 ± 0.36 while in the β -thalassemia trait it was 1.76 ± 0.35 .

The β -thalassemias are the most common disorders of globin chain synthesis in Sicily [1]. During the last 10 years different groups have shown that in β -thalassemias the molecular defect is total (β^0 Th type) or partial (β^+ Th type) depression of normal β -chain synthesis [2-7].

Unlike Ferrara where only the β^0 Th is present, in Sicily β -thalassemia is heterogeneous, and both forms are present [8]. The defect of β -chain synthesis causes a relative increase in the α -chain in both forms, so that the $\alpha/\text{non-}\alpha$ ratio, which is balanced in normal subjects, is unbalanced in the β -thalassemias. In the β -thalassemia trait, this ratio is above 1.50. In homozygotes for β -thalassemia the imbalance is more severe with an $\alpha/\text{non-}\alpha$ ratio above 3 [9].

This work is dedicated to the memory of Prof. F. PARADISO, who first described cardiomegaly in Cooley's disease.

Received: October 3, 1977; accepted: February 15, 1978.

- in alcoholics, cirrhosis, haemochromatosis and pancreatitis. *Am. J. dig. Dis.* 10: 249-258 (1965)
- 12 DELWAIDE, P. A. Mesure de l'absorption digestive du Fe^{59} au moyen d'un «anthropogammamètre» à scintillateur organique. *Nouv. Revue fr. Hémat.* 12: 683-689 (1972)
 - 13 DYMOCK, I. W., GODFREY II E., and WILLIAMS, R. A comparison of methods for the determination of iron absorption using a whole-body counter. *Physica Med. Biol.* 16: 269-273 (1971).
 - 14 FORTU, W. and RUMMEL, J. Iron absorption. *Physiol. Rev.* 53: 724-792 (1973).
 - 15 GROSS, S. J., STUART M. J., SWINDER, P. T. and OSKI, F. A. Malabsorption of iron in children with iron deficiency. *J. Pediatr.* 88: 795-799 (1976).
 - 16 HENRICH, H. C. Intestinal iron absorption in man, method of measurements, dose relationship, diagnostic and therapeutic applications, in HALLBERG, H., WERTH and VANOTTI. Iron deficiency pathogenesis, clinical aspects and therapy pp. 213-298 (Academic Press, London 1970).
 - 17 International Committee for Standardization in Hematology. Proposed recommendations for measurement of serum iron in human blood. *Br. J. Haematol.* 20: 451-453 (1971)
 - 18 JACOB, A. The mechanism of iron absorption. *Clin. Haematol.* 2: 323-337 (1973).
 - 19 LAYRISSE, M. Dietary iron absorption. In KJEF. Iron metabolism and its disorders, pp. 25-33 (Excerpta Medica, Amsterdam 1975)
 - 20 LINDSHEER, W. G., GREENBERG, M. S., MOORE, E. W. and CHALMERS, T. C. Absorption in the proximal small intestine in patients with cirrhosis. *Gastroenterology* 46: 662-670 (1964).
 - 21 MAC CANCE, R. A. and WIDDOWSON, E. M. Absorption and excretion of iron. *Lancet* 1: 680-684 (1937).
 - 22 MAGNUMSON, B. E. O. Iron absorption after antrectomy with gastroduodenostomy. *Scand. J. Haematol.* suppl. 26, pp. 1-111 (1976).
 - 23 MURRAY, M. J. and STEIN, N. Effect of pancreas extracts on Fe^{59} absorption by anemic rats. *J. Lab. clin. Med.* 70: 361-364 (1967)
 - 4 POWELL, L. W. The role of alcoholism in hepatic iron storage disease. *Ann. N. Y. Acad. Sci.* 257: 124-134 (1975)
 - 25 PRICE, D. C., COHN, S. H., WASSERMAN, L. R., REICHSTEIN, P. G. and CROMBIE, E. P. The determination of iron absorption and loss by whole-body counting. *Blood* 20: 517-530 (1962).
 - 26 RAMSAY, V. N. M. The determination of the total binding capacity of serum. *Clinica chim. Acta* 2: 221-226 (1957).
 - 27 SCHIFFER, L. M., PRICE, D. C., CUTNER, J., COHN, S. H. and CROMBIE, E. P. A note concerning the 100 per cent value in iron absorption studies by whole-body counter. *Blood* 23: 757-760 (1964).
 - 28 VAN HOCK, R. and CONRAD, M. E. Iron absorption. Measurement of ingested iron⁵⁹ by a human whole-body liquid scintillation counter. *J. clin. Invest.* 40: 1153-1159 (1961).

A. CELADA, MD Clinique médicale thérapeutique, Hôpital cantonal, CH-1206 Genève (Switzerland)

Table I

Genotype	umber of cases	α /non- α ratio (mean \pm SD)
β^0 Th major	16	4.22 ± 1.88
β^+ Th major	33	3.46 ± 1.36
RGMD	9	1.2 ± 0.36
β -Thalassemia trait	50	1.76 ± 0.35
Normals	30	1.02 ± 0.10

asemia trait with a mean α /non- α ratio of 2.12 ± 0.36 . This difference was statistically significant ($p < 0.01$).

In studying the globin synthesis in thalassemia major we divided the patients into two groups: the first consisted of 16 children and was characterized by absence of β -chain synthesis (β^0 Th). The second group of 33 children had measurable, although very low levels of β -chain synthesis (β^+ Th). This could be the phenotypic expression of either homozygous β^0 Th or double heterozygosity for β^0/β^+ . Both groups showed severe imbalance: the α /non- α ratio in the first group was 4.22 ± 1.88 and in the second was 3.46 ± 1.36 . However, this difference was not statistically significant ($p < 0.10$).

On the other hand, the difference between RGMD and β -thalassemia major was statistically significant ($p < 0.01$).

Discussion

β -Thalassemia comprises a group of different clinical and hematological pictures. β -Thalassemia trait is usually clinically asymptomatic; RGMD presents a picture of intermediate severity; thalassemia major is the most severe form of this syndrome and requires frequent blood transfusions and results in death before the second decade of life.

In figure 1 the α /non- α ratio of normal subjects and patients with these three forms of β^0 Th have been compared. These values decrease gradually from thalassemia major to the thalassemia trait.

The imbalance observed in RGMD has been found to be greater than in healthy carriers of β -thalassemia and less than in thalassemia major. In fact our patients have mild disease with moderate chronic hemolytic and

In this paper we compare the degree of imbalance in the β Th with the β^+ Th and show the frequency of the two forms in Sicilian β -thalassemic subjects. We have also studied hemoglobin synthesis in the Rietti Greppi Micheli disease (RGMD). This latter form is a β -thalassemia of intermediate severity and it has an interesting genetic picture with one parent being a carrier of the β -thalassemia trait and the other being hematologically and clinically normal [10-14]. In our cases the normal parent showed also a balanced hemoglobin synthesis.

Materials and Methods

We studied 50 carriers of the β -thalassemia trait, 9 patients with RGMD and 49 with β thalassemia Major. All the cases studied were unselected and came from South-East Sicily. A group of 30 normal children were used as a control. In all cases the disease was diagnosed by genetic studies and hematological data.

Routine hematological tests were performed using standard methods [15]. Estimation of HbF was made by the method of BETKE *et al* [16]. Hemoglobin was analyzed by electrophoresis on cellulose acetate strips in glycine buffer at pH 9.0. Quantitative analysis of HbA₂ was carried out by elution of the hemoglobin fractions from cellulose acetate strips [17].

Globin chain synthesis study was performed from 24 to 53 days after the last transfusion by incubating washed red cells with ³H-leucine. After 7 h the incubation was stopped and the cells were washed and lysed with distilled water. Globin was prepared by acid acetone precipitation by the method of ROSSI-FANELLI [18], and the globin chains were separated chromatographically on CM-cellulose according to the method originally described by CLEGG *et al* [19]. Optical density was continuously measured with an LKB Uvicord at 80 nm. The radioactivity of each fraction was measured with a Nuclear Chicago Mark II scintillation counter. The total radioactivity ratio of ³H α /non- α chain synthesis was calculated.

Routine hematological tests in patients with β -thalassemia major were made at the time of diagnosis before transfusion.

Results

The results of globin chain synthesis are summarized in table I.

Subjects with the β -thalassemia trait demonstrate mild imbalance of chain synthesis: the mean α /non- α ratio is 1.76 ± 0.35 SD. In normal subjects, the globin synthesis is always balanced with the mean α /non- α ratio being 1.02 ± 0.10 . This difference is statistically significant ($p < 0.01$).

In our patients with RGMD imbalance was greater than in the β thal

Table I

Genotype	Number of cases	$\alpha/\text{non-}\alpha$ ratio (mean \pm SD)
β^0 Th major	16	4.22 ± 1.88
β^+ Th major	33	3.46 ± 1.36
RGMD	9	2.12 ± 0.36
β -Thalassemia trait	50	1.76 ± 0.35
Normals	30	1.02 ± 0.10

asemia trait with a mean $\alpha/\text{non-}\alpha$ ratio of 2.12 ± 0.36 . This difference was statistically significant ($p < 0.01$).

In studying the globin synthesis in thalassemia major we divided the patients into two groups: the first consisted of 16 children and was characterized by absence of β -chain synthesis (β^0 Th). The second group of 33 children had measurable, although very low levels of β -chain synthesis (β^+ Th). This could be the phenotypic expression of either homozygous β^+ Th or double heterozygosity for β/β^+ . Both groups showed severe imbalance; the $\alpha/\text{non-}\alpha$ ratio in the first group was 4.22 ± 1.88 and in the second was 3.46 ± 1.36 . However this difference was not statistically significant ($p < 0.10$).

On the other hand, the difference between RGMD and β -thalassemia major was statistically significant ($p < 0.01$).

Discussion

β -Thalassemia comprises a group of different clinical and hematological pictures. β -Thalassemia trait is usually clinically asymptomatic; RGMD presents a picture of intermediate severity; thalassemia major is the most severe form of this syndrome and requires frequent blood transfusions and results in death before the second decade of life.

In figure 1 the $\alpha/\text{non-}\alpha$ ratio of normal subjects and patients with these three forms of β^+ Th have been compared. These values decrease gradually from thalassemia major to the thalassemia trait.

The imbalance observed in RGMD has been found to be greater than in healthy carriers of β -thalassemia and less than in thalassemia major: in fact our patients have mild disease with moderate chronic hemolytic and



Fig 1 The ratios of α /non- α chains in individuals with different types of β -thalassaemia.

nia, scleral icterus and splenomegaly. The α /non- α ratio corresponds to that reported in the literature for thalassaemia of intermediate severity [9].

This term has been introduced to indicate all those forms of thalassaemia of intermediate clinical and hematological picture between thalassaemia major and the asymptomatic carrier: these forms are a genetically heterogeneous group which generally result from the interaction between two different thalassaemic anomalies [9].

FRIEDMAN *et al* [20] have recently described patients with genetic, clinical and hematological pictures identical to that of our patients with RGMD in whom the imbalance of β -globin chain synthesis is similar to our subjects. These authors consider these subjects to be heterozygous for

Table II Hematological findings in β^0 Th, β^+ Th and RGMD (mean \pm SD)

	Age	Number of cases	Hb g/100 ml	RBC 10^6 mm ³	MCHC g/100 ml	MCH pg	MCV μ m ³	HbA ₂ %	HbF %
β^0 Th major	2.1 ± 2.8	18	5.5 ± 2.38	2.49 ± 1.80	26.51 ± 1	21.45 ± 1.30	71 ± 0.40	1.3 ± 0.5	80 ± 1.10
β^+ Th major	1.11 ± 3	33	5.39 ± 2.46	2.60 ± 2	26.17 ± 0.5	20.5 ± 0.90	71 ± 1	1.4 ± 0.6	81 ± 0.56
RGMD	11.3 ± 3	9	7.9 ± 2	3.08 ± 1	28 ± 1	20.7 ± 1	75 ± 3	4.75 ± 0.8	9.6 ± 2.4

β -thalassemia of unusual severity Italian authors [10-14] have seen that the heterozygous condition can appear in many different clinical and hematological forms. The most common is the healthy carrier while RGMD which is a form of intermediate severity is much rarer. The disease in the heterozygous β -thalassemia condition has been explained by two different hypotheses, (1) greater expressivity of the gene [13] and (2) action of modifier genes (derived from an apparently normal parent) enhancing the expression of β -Th gene [21].

In our unselected thalassemia population, about 30% have been found to be β^0 Th. This frequency is lower than in the Ferrara β^0 Th where it is 95% [5]. The mean α /non- α ratio in patients suffering from β^0 Th was slightly lower than in those suffering from β^+ Th, but this difference was not statistically significant. In the former the β -gene activity at the protein synthesis level is not demonstrable, while in the latter it is assessable and HbA is produced, but in both forms, the imbalance and the hematological findings are the same (table II).

So the absent synthesis of β -chain in β^0 Th homozygotes does not seem to be associated with an increased synthesis of HbF as seen in homozygous $\delta\beta$ -thalassemia and HPFH. On the other hand, all the three conditions (β^0 Th, $\delta\beta$ -Thalassemia and HPFH) have the absence of β -chain synthesis in common. Nonetheless, in some cases of homozygous HPFH there is neither anemia, nor other clinical abnormalities: the α /non- α ratio is balanced [22, 24]. The γ -chain synthesis appears to compensate fully for the absence of β -chain synthesis. In other cases of HPFH as in homozygous $\delta\beta$ thalassemia there is moderate anemia and very mild splenomegaly [25-27, 29, 30]. The α /non- α ratio is mildly unbalanced. The γ -chain synthesis compensates quite well for the absence of β -chain



Fig 1 The ratios of α /non- α chains in individuals with different types of β -thalassemia.

mia, scleral icterus and splenomegaly. The α /non- α ratio corresponds to that reported in the literature for thalassemia of intermediate severity [9].

This term has been introduced to indicate all those forms of thalassemia of intermediate clinical and hematological picture between thalassemia major and the asymptomatic carrier: these forms are a genetically heterogeneous group, which generally result from the interaction between two different thalassemic anomalies [9].

FRIEDMAN *et al* [20] have recently described patients with genetic, clinical and hematological pictures identical to that of our patients with RGMD, in whom the imbalance of β -globin chain synthesis is similar to our subjects. These authors consider these subjects to be heterozygous for

Table II Hematological findings in β^0 Th, β^+ Th and ROMD (mean \pm SD)

	Age	Number of cases	Hb g/100 ml	RBC $10^6/\text{mm}^3$	MCHC g/100 ml	MCH pg	MCV μm^3	HbA %	HbF %
β^0 Th major	2.1 ± 2.8	18	5.5 ± 2.38	2.49 ± 1.80	26.51 ± 1	21.45 ± 1.30	71 ± 0.40	1.3 ± 0.5	80 ± 1.10
β^+ Th major	1.11 ± 3	33	5.39 ± 2.46	2.60 ± 2	26.17 ± 0.5	20.5 ± 0.90	71 ± 1	1.4 ± 0.6	III ± 0.56
ROMD	11.3 ± 3	9	7.9 ± 2	3.08 ± 1	28 ± 1	20.7 ± 1	75 ± 3	4.75 ± 0.8	9.6 ± 2.4

β -thalassemia of unusual severity Italian authors [10-14] have seen that the heterozygous condition can appear in many different clinical and hematological forms. The most common is the healthy carrier while ROMD which is a form of intermediate severity is much rarer. The disease in the heterozygous β -thalassemia condition has been explained by two different hypotheses. (1) greater expressivity of the gene [13] and (2) action of modifier genes (derived from an apparently normal parent) enhancing the expression of β -Th gene [21].

In our unselected thalassemia population, about 30% have been found to be β^0 Th. This frequency is lower than in the Ferrara β^0 Th where it is 95% [5]. The mean $\alpha/\text{non-}\alpha$ ratio in patients suffering from β^0 Th was slightly lower than in those suffering from β^+ Th, but this difference was not statistically significant. In the former the β -gene activity at the protein synthesis level is not demonstrable, while in the latter it is assessable and HbA is produced, but in both forms, the imbalance and the hematological findings are the same (table II).

So the absent synthesis of β -chain in β^0 Th homozygotes does not seem to be associated with an increased synthesis of HbF as seen in homozygous $\delta\beta$ -thalassemia and HPFH. On the other hand, all the three conditions (β^0 Th, $\delta\beta$ -Thalassemia and HPFH) have the absence of β -chain synthesis in common. Nonetheless, in some cases of homozygous HPFH there is neither anemia, nor other clinical abnormalities, the $\alpha/\text{non-}\alpha$ ratio is balanced [22, 24]. The γ -chain synthesis appears to compensate fully for the absence of β -chain synthesis. In other cases of HPFH as in homozygous $\delta\beta$ -thalassemia there is moderate anemia and very mild splenomegaly [25-27, 29, 30]. The $\alpha/\text{non-}\alpha$ ratio is mildly unbalanced. The γ -chain synthesis compensates quite well for the absence of β -chain

synthesis. On the contrary in β^0 Th there is clinical and hematological severity. The absence of β -chain synthesis appears to be insufficiently compensated by γ -chain synthesis.

In both $\delta\beta$ -thalassemia and HPHF there is a deletion of β -genes (and possibly of δ -genes) [29-30]; on the contrary in both β^+ Th and β^0 Th where the severity and α /non- α ratio are almost identical there is no deletion of β and δ -genes [31-32].

It seems that the compensating γ -chain synthesis is associated with the absence of β -genes rather than with the absence of β and δ polypeptide chain synthesis.

Acknowledgment

We are grateful to Drs. R. CURRERI and R. TESTA for their technical assistance.

References

1. RUSSO, G. e MOLLIKA, R. Thalassemia ed emoglobine anomale nella Sicilia occidentale: ricerche sui campioni d' popolazione infantile non selezionata e sul materiale casistico della Clinica Pediatrica di Palermo, Riv. Ped. Siciliana 2/ 180-187 (1966).
2. HEYWOOD, J. D., KARON, M. and WEISSMAN, S. Amino acid incorporation into alpha and beta chains of hemoglobin by normal and thalassemic reticulocytes. Science 146: 530-531 (1964).
3. WEATHERALL, D. J., CLEGG, J. B. and NAUGHTON, M. A. Globin synthesis in β -thalassaemia. An *in vitro* study. Nature Lond. 208: 1061-1062 (1965).
4. BANK, A. and MARKE, P. A. Excess alpha chain synthesis relative to beta chain synthesis in thalassaemia major and minor. Nature Lond. 212: 1198-1200 (1966).
5. BARGELLER, A., PONTREMOLI, S., and CONCONI, F. Absence of beta-globin synthesis and excess of alpha globin synthesis in homozygous beta-thalassaemia. Eur. J. Biochem. 1: 73-79 (1967).
6. MODELL, C. B., LATTIE, A., STEADMAN, J. H. and HUEBNER, H. R. Haemoglobin synthesis in β -thalassaemia. Br. J. Haemat. 17: 485-501 (1969).
7. RUSSO, G., MUSUMECI, S., SCHILLER, G., D'AGATA, A. e PIZZARTILLI, G. Sintesi *in vitro* delle catene polipeptidiche della globina nelle talassemie ed in alcune emoglobinopatie. Atti Soc. Ital. Ematol. 1: 197-205 (1974).
8. RUSSO, G., MOLLIKA, F., PAVONE, L., MUSUMECI, S., and BAGLIONI, C. Genetic implications of the interaction of two types of beta-thalassaemia genes in a patient with thalassaemia major. Blood 42: 763-769 (1973).
9. WEATHERALL, D. J. and CLEGG, J. B. The thalassaemia syndromes. 2nd ed. (Blackwell Oxford 1972).

- 10 RIETTI, F.: Haemolytic anemias with increased osmotic resistance of the erythrocytes. *Acta med. scand.* 125: 451-462 (1946).
- 11 GREPPI, E.: Itero emolitico familiare con aumento dell' resistenza dei globuli rossi. *Minerva med.* 8: 1-10 (1928).
- 12 MICHELLI, F., PEXATE, F. e MONSOLLANO, L. G.: Ulteriori ricerche sulla anemia ipocromica splenomegalica con poichlocitosi. *Atti Soc. ital. Ematol. Haematologica* 16, suppl., pp. 10-20 (1933).
- 13 ITRIO, L.: Genetica della talassemia e della drepanocitosi. *Atti 3 Congr. Igiene Medicina Mediterranea, Palermo* 1951.
- 14 RUSSO, G.: Genetica: classificazione delle talassemie. *Prosp. Pediat.* 11: 363-378 (1973).
- 15 DACEY, J. V. and LEWIS, S. M.: *Practical haematology* 4th ed (Churchill, London 1968).
- 16 BITEK, K., MARTI, H. R. and SCHLICHT, I.: Estimation of small percentages of foetal haemoglobin. *Nature, Lond.* 184: 1877-1878 (1959).
- 17 DI STEFANO, G. MARCELLENI, M. L.: La determinazione dell'emoglobina A₂ mediante elettroforesi su acetato di celloso. *Riv. Ped. Siciliana* 24: 36-45 (1969).
- 18 ROMI FANELLI, A. R., ANTONIOLI, M. and CAPUTO, A.: Studies on the structure of haemoglobin. I. Physicochemical properties of human globin. *Biochim. biophys. Acta* 30: 608-615 (1958).
- 19 CLEGG, J. B., NAUGHTON, M. A. and WEATHERALL, D. J.: Abnormal human haemoglobins. Separation and characterization of the alpha and beta chains by chromatography and the determination of re- new variants, Hb Chesapeake and Hb J (Bangkok). *J. molec. Biol.* 19: 91-108 (1966).
- 20 FRIEDMAN, S. H., OMICILU, S., LUDDY, R., and SCHWARTZ, E.: Heterozygous beta-thalassemia of unusual severity. *Br. J. Haemat.* 32: 65-77 (1976).
- 21 MURATORE, F.: Lemolia nelle enteropatie con macrofiliisopochidlocitosi iperresistente tipo Rietti Greppi-Micheli. *Riv. med.* 61: 496 (1948).
- 22 NITTA, C. L., NIAZZI, G. A., FORD, S. and BANT, A.: Balanced globin chain synthesis in hereditary persistence of fetal hemoglobin (HPHF). *J. clin. Invest.* 54: 433-438 (1974).
- 23 SOFRONIADOU, K., WOOD, W. G., NITE, P. E., and STAMATOYANNIDIS, G.: Globin chain synthesis in the greek type (γ) of hereditary persistence of fetal hemoglobin. *Br. J. Haemat.* 29: 137-148 (1975).
- 24 FRIEDMAN, S., SCHWARTZ, E., AHERN, E. and AHERN, V.: Variations in globin chain synthesis in hereditary persistence of fetal haemoglobin. *Br. J. Haemat.* 32: 357-364 (1976).
- 25 RUSSO, G., MUSUMECI, S., SCHILIND, G., D'AGATA, A., and PIZZARELLI, G.: Haemoglobin synthesis in delta-beta thalassemia. *Atti Vth Congr. Microcitrom, Comerio* 1973, pp. 101-106.
- 26 FOWKE, B. G., HELLMAN, D. G., LAZARUS, H., BARELL, E. F., BENTZ, E. S., CASEY, C. T., HUFFMAN, T. H. S., SCHROEDER, W. A. and HOUTMAN, O.: Absence of messenger RNA and gene DNA for β -globin chains in hereditary persistence of fetal hemoglobin. *Cell* 7: 323-329 (1976).
- 27 CHARACTIS, S., CLEGG, J. B. and WEATHERALL, D. J.: The negro variety of hered-

- itary persistence of fetal haemoglobin is a mild form of thalassaemia. *Br J Haemat.* 34: 527-534 (1976)
- 28 KAN Y W, HOLLAND, J P, DOZY A M, CHARACH S and KAZAZIAN H H. Deletion of the globin structure gene in hereditary persistence of foetal haemoglobin. *Nature, Lond.* 258: 162-163 (1975)
- 29 RAMIREZ, F, O'DONNELL, J V., MARKS, P A., BANK, A., MUSUMECI, S., SCHILLER, G, PIZZARELLI, G, RUSSO, G, LUPPIS, B and GAMRINO, R.. Abnormal or absent mRNA in β^0 Th Ferrara and gene deletion in delta-beta thalassaemia. *Nature Lond.* 263: 471-473 (1976).
- 30 OTTOLENGHI, S., COMI, P., GIOLIONI, B., TOLSTOSHEV, P., LANYON, W G., MITCHELL, E. S., WILLIAMSON, R., RUSSO, G., MUSUMECI, S., SCHILLER, G., TSTRAKIS, G A., CHARACH, S., WOOD, W G and WEATHERALL, D J.. β^0 thalassemia is due to a gene deletion. *Cell* 9: 71-80 (1976).
- 31 RAMIREZ, F, O'DONNELL, J V, MUSUMECI, S, RUSSO, G and BANK, A. Presence of β globin genes in β^0 thalassemia. *Clin. Res.* 3: 583A (1975)
- 32 TOLSTOSHEV, P., MITCHELL, J., LANYON, G, MASERA, G, MODILL, B, WEATHERALL, D J and CLEGG, J B. Presence of gene for β globin in homozygous β^0 thalassemia. *Nature Lond* 259: 95-98 (1976)
- 33 PARADISO, P. Su alcuni casi di anemia con eritroblastosi ed alterazioni scheletriche a base prevalentemente osteoporotica. *Ped. at. med. prat.* 9: 1-2 (1934)

Relationship of Density Distribution and Pyruvate Kinase Electrophoretic Pattern of Erythrocytes in Sickle Cell Diseases and Other Disorders¹

SUZUMU ODA, ETSUKO ODA and KOICHI R. TANAKA²

Department of Medicine, Harbor General Hospital, Torrance, Calif., and
UCLA School of Medicine, Los Angeles, Calif.

Key Words: Red cell density distribution Pyruvate kinase electrophoresis
Sickle cell diseases

Abstract The density distributions of red cells from sickle cell disorders and other hematological diseases were determined. In sickle cell anemia there was an increase in the proportion of cells in both the heaviest and lightest fractions. In hemoglobin SC disease, small fraction was shifted to heavier cells. Sickle cell trait blood had normal density pattern. In hereditary spherocytosis an increase in the number of heavy cells was observed. Thin-layer polyacrylamide gel electrophoresis of red cell pyruvate kinase demonstrated that the pyruvate kinase electrophoretic pattern was related to density distribution.

Introduction

The density distribution of blood from patients with sideroblastic anemia [7] sickle cell anemia and sickle cell trait [1] has been described subsequently to the introduction of a simple method by DAMON and MARIKOVSKY [10]. A modification of this method has been reported to be an useful tool to evaluate blood disorders [18]. To date, however the density distribution of red cells in various blood disorders has not been extensively studied.

This work was supported by grant AM-14898 from the National Institute of Arthritis, Metabolism, and Digestive Diseases.

The authors wish to thank Mr. HUDO GALMARINI and Mrs. MIRYA GALMARINI for expert technical assistance.

It is well established that the specific gravity of red cells increases with cell age [4 6 14 20]. Recently by electrophoresis of human erythrocyte pyruvate kinase (PK R) two bands have been demonstrated [5 16 17 19] which NAKASHIMA [16] has designated PK R₁ and PK R₂. Younger cells have more PK R₁ and PK R₂ increases as the cells age.

In this paper the density distributions of red cell populations from sickle cell disease patients as well as from other hematological disorders are described. Also a relationship between density distribution and electrophoretic bands of PK R is shown.

Materials and Methods

Venous blood specimens were collected in heparin or EDTA from normal volunteer subjects and patients at Harbor General Hospital and used within 5 h. Reticulocyte counts and MCHC were determined by standard methods [7]. Sickle cell disease was diagnosed by the presence of sickling and hemoglobin electrophoresis with cellulose acetate (Gelman Instrument Company, Michigan).

Determinations of red cell density distributions were made by a modification [18] of the method of DANON and MARIKOVSKY [10], except that one separation fluid was used for each capillary glass tube as in the original method. The tube was centrifuged in a microcapillary centrifuge (Clay Adams, Inc., New York) at 12,000 rpm for 10 min.

For PK electrophoresis the hemolysate was prepared by a previously described method [17]. Thin-layer polyacrylamide gel electrophoresis and staining of PK were performed using the method of IMAMURA and TANAKA [13], except that electrophoresis was carried out at 10 V/cm for 6–8 h. Phosphoenolpyruvate, fructose-1,6-diphosphate, adenosine diphosphate and N N N N tetramethylethylenediamine were purchased from Sigma, lactate dehydrogenase and NADH from Calbiochem, and acrylamide, bis-(N,N-methylene-bis-acrylamide), and ammonium persulfate from Isolab.

Results

The mean (± 2 SD) of red cell density distributions from normal individuals is shown in figure 1. The normal range was from 1.122 to 1.080 in both males and females, but the male distribution is shifted slightly toward increased density.

The density distribution curve in sickle cell anemia (fig. 2) indicates that a small part of the red cell population ($8.5 \pm 4.0\%$ mean \pm SD) has a very high density (greater than 1.122) while another small portion of the population shows a lighter than normal range. There was no apparent



Fig 1 Normal range of red cell density distribution (10 males, 10 females).

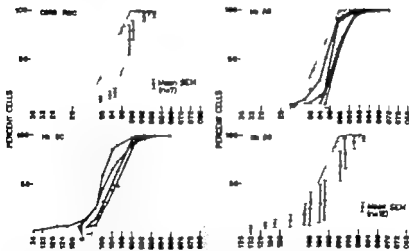


Fig 2 Red cell density distribution of cord blood (top left), sickle cell trait (top right), hemoglobin SC disease (bottom left), and sickle cell anemia (bottom right). The normal range (shaded) includes normal males and females.

difference in the density curves of sickle cell anemia patients in crisis compared to the same patients in remission. In hemoglobin SC disease (fig. 2) there was a left shift of the density distribution curve, but the percentage of heavy erythrocytes was smaller than in sickle cell anemia. Reticulocyte counts from hemoglobin SC disease patients were 11.6, 3.8,

It is well established that the specific gravity of red cells increases with cell age [4 6 14 20]. Recently by electrophoresis of human erythrocyte pyruvate kinase (PK R) two bands have been demonstrated [5 16 17 19] which NAKASHIMA [16] has designated PK R₁ and PK R₂. Younger cells have more PK R₁ and PK R₂ increases as the cells age.

In this paper the density distributions of red cell populations from sickle cell disease patients as well as from other hematological disorders are described. Also, a relationship between density distribution and electrophoretic bands of PK R is shown.

Materials and Methods

Venous blood specimens were collected in heparin or EDTA from normal volunteer subjects and patients at Harbor General Hospital and used within 5 h. Reticulocyte counts and MCHC were determined by standard methods [7]. Sickle cell disease was diagnosed by the presence of sickling and hemoglobin electrophoresis with cellulose acetate (Gelman Instrument Company, Michigan).

Determinations of red cell density distributions were made by a modification [18] of the method of DANON and MARIKOVSKY [10] except that one separation fluid was used for each capillary glass tube as in the original method. The tube was centrifuged in a microcapillary centrifuge (Clay Adams, Inc., New York) at 12,000 rpm for 10 min.

For PK electrophoresis the hemolysate was prepared by a previously described method [17]. Thin layer polyacrylamide gel electrophoresis and staining of PK were performed using the method of IMAMURA and TANAKA [13] except that electrophoresis was carried out at 10 V/cm for 6–8 h. Phosphoenolpyruvate, fructose 1,6-diphosphate, adenosine diphosphate and N,N,N,N-tetramethylethylenediamine were purchased from Sigma, lactate dehydrogenase and NADH from Calbiochem, and acrylamide, bis-(N,N-methylene-bis-acrylamide), and ammonium persulfate from Isolab.

Results

The mean (± 2 SD) of red cell density distributions from normal individuals is shown in figure 1. The normal range was from 1.122 to 1.080 in both males and females, but the male distribution is shifted slightly toward increased density.

The density distribution curve in sickle cell anemia (fig. 2) indicates that a small part of the red cell population ($8.5 \pm 4.0\%$ mean \pm SD) has a very high density (greater than 1.122) while another small portion of the population shows a lighter than normal range. There was no apparent



Fig 4 Polyacrylamide gel electrophoresis of PK-R. 1 = Sickle cell anemia, 2 = normal control 3 = pernicious anemia (after treatment).



Fig 5 Polyacrylamide gel electrophoresis of PK-R. 1 = Hemoglobin SC disease, 2 = pernicious anemia (before treatment) 3 = autoimmune hemolytic anemia, 4 = normal control 5 = pernicious anemia (before treatment).

cells. Thalassemia minor and sickle cell thalassemia showed a normal density distribution.

1 case of pernicious anemia showed a normal density curve before treatment and an increase in the light population after treatment (bottom half of figure 3). Reticulocyte counts before and after treatment were 2.4 and 24.6%, respectively. A case of autoimmune hemolytic anemia with a 69.9% reticulocyte count showed a marked increase in the light population, and some of the red cells were outside of the normal range on the heavy side. The density distribution curves (not shown) in chronic liver or

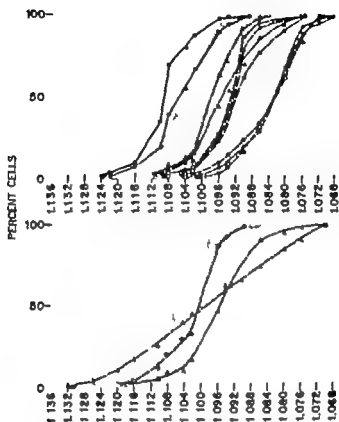


Fig 3 Red cell density distribution of patients with (top half) iron deficiency anemia (O), HS (●) sideroblastic anemia (Δ) and thalassemia minor (\blacktriangle) and with (bottom half) pernicious anemia (O) before treatment Δ after treatment and autoimmune hemolytic anemia (\blacktriangle).

1.0, 0.8 and 0.8%. Sickle cell trait cells showed a normal density curve (fig 2). In cord blood samples (fig 2) there was a slight increase in the light population but otherwise these erythrocytes were within the normal range.

As shown in the top half of figure 3, all 6 cases of iron deficiency anemia had density distribution curves shifted to the right with the degree of shift related to the severity of the iron deficiency. 1 case of sideroblastic anemia revealed a pattern similar to that of mild iron deficiency anemia. The density distribution of 1 of the 2 hereditary spherocytosis (HS) patients (2 years after splenectomy) was shifted to the left. The other patient with HS with a reticulocytosis of 9% showed no increase in the light population of cells as in other anemias, but rather a relative increase in heavy



Fig. 4. Polyacrylamide gel electrophoresis of PK-R. 1 = Sickle cell anemia; 2 = normal control; 3 = pernicious anemia (after treatment).



Fig. 5. Polyacrylamide gel electrophoresis of PK-R. 1 = Hemoglobin SC disease; 2 = pernicious anemia (before treatment); 3 = autoimmune hemolytic anemia; 4 = normal control; 5 = pernicious anemia (before treatment).

cells. Thalassemia minor and sickle cell thalassemia showed a normal density distribution.

1 case of pernicious anemia showed a normal density curve before treatment and an increase in the light population after treatment (bottom half of figure 3). Reticulocyte counts before and after treatment were 2.4 and 24.6%, respectively. A case of autoimmune hemolytic anemia with a 69.9% reticulocyte count showed a marked increase in the light population, and some of the red cells were outside of the normal range on the heavy side. The density distribution curves (not shown) in chronic liver or

renal disease patients were normal except for 2 cases of obstructive jaundice which showed a right shift.

PK electrophoresis with thin layer polyacrylamide gel showed a marked increase in PK R_1 in all sickle cell anemia patients (fig. 4). The PK pattern of hemoglobin SC disease patients was variable. Of 4 SC patients, the one with the heaviest cells had predominantly PK R_2 (fig. 5). Sickle cell trait red cells contained more PK R_2 than PK R_1 , similar to that of the normal control. The red cells of a pernicious anemia patient showed a marked increase in PK R_1 after treatment (fig. 4), compared to mostly PK R_2 before treatment (fig. 5).

The MCHC of various blood disorders was as follows: sickle cell anemia 33.2, 33.3, 33.0, 36.0, 35.2, 32.4, 34.9, 31.1, 32.2, 36.2, 31.7 and 34.2; hemoglobin SC disease 32.8, 34.9, 35.6, 35.3 and 34.6; sickle cell trait 32.8, 32.6, 31.7 and 33.1; iron deficiency anemia 29.9, 30.4, 30.5, 30.7, 31.4 and 31.9; sideroblastic anemia 30.9; HS 38.0 and 37.8; cord blood 34.1, 34.7, 34.2, 30.2, 32.2, 31.9 and 32.8.

Discussion

Our results confirm a previous report [22] that normal female subjects have lighter red cell populations than males. Since normal females are frequently iron-deficient [21], erythrocytes from apparently normal females may be lighter because of borderline iron deficiency.

In sickle cell anemia the increase in light cells is related to the degree of reticulocytosis, which correlates with a marked increase in PK R_1 , as shown by electrophoresis. This relationship between density distribution and the quantity of each of the PK bands was confirmed by the young red cells of autoimmune hemolytic anemia and recently treated pernicious anemia, showing an increase in the proportion of light cells and an increase in PK R_1 , as previously described [17].

Irreversibly sickled cells (ISC) are the heaviest cells in sickle cell anemia [1, 8]. The transformation of non ISC into ISC presumably begins soon after cell release from the marrow [3]. The mean age of erythrocytes in sickle cell anemia is very young despite the presence of a heavier than normal subpopulation.

The contribution of increased HbF to the altered density distribution curve in sickle cell anemia was evaluated by examining the density distribution of cord blood which contains a high percentage of HbF. All curves

of cord blood were consistent with reticulocytosis, that is, an increase in light cells. Therefore, the presence of HbF itself in sickle cell anemia does not appear to affect the density distribution curve.

Hemoglobin SC disease showed an increase in heavy red cells and a small number of red cells whose density was outside the normal range. The red cells of hemoglobin SC disease may have less water as has been reported in hemoglobin C disease [15] and sickle cell anemia [12]. An increase in heavy red cells might also be partly accounted for by an increased population of older red cells because PK R_s was very prominent. However even in the hemoglobin SC patient with the lightest red cell population, the curve was shifted toward heavy cells if the high reticulocytosis (11.6%) in this patient is considered. The slightly heavier density curve of the red cells of hemoglobin SC disease may be due to higher than normal MCHC. In fact, our data suggest that MCHC correlates with the density distribution curve. Red cells with high MCHCs, as in sickle cell anemia, especially ISC, and microspherocytes of HS, had a heavy density distribution curve. In contrast, iron deficiency anemia associated with a low MCHC showed a light density distribution curve.

A change in plasma osmolarity or jaundice might modify the density distribution curve. However in chronic renal or liver disease with increased plasma osmolarity or jaundice, the density distribution did not change beyond the normal range (mean \pm 2 SD), suggesting that these factors do not affect the distribution. On the other hand, when target cells are seen, the distribution is frequently abnormal. In obstructive jaundice cells are lighter than normal. In hemoglobin SC disease the cells are heavier or normal, and in thalassemia minor they are generally of normal density despite the presence of many target cells. This may be the result of different mechanisms of target cell formation. In obstructive jaundice, the lipid content of the membrane increases, resulting in an increase in the surface area without remarkable change in cell protein [9]. This may decrease the density of the red cells. It has been suggested that a change in the distribution of hemoglobin in HbC disease may bring about target cell formation [11]. This redistribution should not bring about a change in density. Target cells in hemoglobin SC disease are probably formed as in HbC disease. The normal red cell density seen in thalassemia minor [18] may be due to the fact that the thin, small cells of thalassemia minor have normal MCHCs, resulting in cells of normal density. Thus the density distribution of red cells illustrates abnormalities of red cells themselves as well as the heterogeneity of the red cell population.

References

- 1 ABRAHAM, E. C. WALKER, D. GRAVELY M. and HUISMAN T. H. J. Minor hemoglobins in sickle cell anemia, β thalassemia, and related conditions. a study of red cell fractions isolated by density gradient centrifugation. *Biochem. Med.* 13 56-77 (1975).
- 2 BEN BASSAT I. BROK SIMONI, F. and RAMOT B. Evidence for abnormal red cell population in refractory sideroblastic anemia. *Blood* 35 453-461 (1970).
- 3 BERTLES, J. F. and MILNER, P. F. A. Irreversibly sickled erythrocytes a consequence of the heterogeneous distribution of hemoglobin types in sickle-cell anemia. *J. clin. Invest.* 47 1731-1741 (1968).
- 4 BISHOP C. and PRENTICE, T. C. Separation of rabbit red cells by density in a bovine serum albumin gradient and correlation of red cell density with cell age after in vivo labeling with ^{59}Fe . *J. cell comp. Physiol.* 67 197-208 (1966).
- 5 BLUME, K. G. LÖBER, G. W. RÖDIGER, H. W. and SCHALHORN A. Pyruvate kinase in human erythrocytes. *Lancet* i 529-530 (1968).
- 6 BROK, F., RAMOT B., ZWANO E., and DANON, D. Enzyme activities in human red blood cells of different age groups. *Israel J. med. Sci.* 2 291-296 (1966).
- 7 CARTWRIGHT G. E. *Diagnostic laboratory hematology* p 109 (Grune & Stratton, New York 1968).
- 8 CHEN, S. USAMI, S. and BERTLES, J. F. Abnormal rheology of oxygenated blood in sickle cell anemia. *J. clin. Invest.* 49 623-634 (1970).
- 9 COOPER, R. A. Lipids of human red cell membrane: normal composition and variability in disease. *Semin. Hematol.* 7 296-322 (1970).
- 10 DANON D. and MARIKOVSKY Y. Determination of density distribution of red cell population. *J. Lab. clin. Med.* 64 668-674 (1964).
- 11 EASLEY A. F. and GABUZDA, T. G. *Pathophysiology of blood*, p 73 (Saunders, Philadelphia 1975).
- 12 HELLERSTEIN S. and BUNTHARUNGRON T. Erythrocyte composition in sickle-cell anemia. *J. Lab. clin. Med.* 83 611-624 (1974).
- 13 IMAMURA, K. and TANAKA, T. Multimolecular forms of pyruvate kinase from rat and other mammalian tissues. *J. Biochem., Tokyo* 71 1043-1051 (1972).
- 14 MARKE, P. A. JOHNSON, A. B. and HIRSCHBERG, E. Effect of age on the enzyme activity of human erythrocytes. *Proc. natn. Acad. Sci. USA* 44 529-536 (1958).
- 15 MURPHY J. R. Hemoglobin CC disease rheological properties of erythrocytes and abnormalities in cell water. *J. clin. Invest.* 47 1483-1495 (1968).
- 16 NAKASHIMA, K. Further evidence of molecular alteration and aberration of erythrocyte pyruvate kinase. *Clinica chim. Acta* 55 45-254 (1974).
- 17 NAKASHIMA, K., MIWA, S., ODA, S., TANAKA, T., IMAMURA, K. and NISHITANI, T. Electrophoretic and kinetic studies of mutant erythrocyte pyruvate kinases. *Blood* 43 537-548 (1974).
- 18 NAKASHIMA, K., ODA, S., and MIWA, S. Red cell density in various blood disorders. *J. Lab. clin. Med.* 82 297-302 (1973).
- 19 PAGLIA, D. E. and VALENTINE, W. N. Evidence for molecular alteration of pyruvate kinase as a consequence of erythrocyte aging. *J. Lab. clin. Med.* 76 202-212 (1970).

- 20 PROMELLI, S. LUDWIKY, G. and WASSERMAN, L. R.. The mechanism of red cell aging. I. Relationship between cell age and specific gravity evaluated by ultracentrifugation in a discontinuous density gradient. *J. Lab. clin. Med.* 69 659-674 (1967).
- 21 SCOTT, D. E. and PATERCHARD, J. A.. Iron deficiency in healthy young college women. *J. Am. med. Ass.* 199 897-900 (1967).
- 22 WINTROW, M. M.. *Clinical hematology* p. 122 (Lea & Febiger Philadelphia 1974).

Functional Heterogeneity of Transferrin-Bound Iron

Iron Uptake by Cell Suspensions from Bone Marrow and Liver and by Cell Cultures of Fibroblasts and Lymphoblasts

N J VERHOEF M. J KOTTENIAGEN H J M MULDER,
P J NOORDELOOS and H LEUNSE

Department of Chemical Pathology Faculty of Medicine, Erasmus University
Rotterdam, Rotterdam

Key Words. Bone marrow Cell cultures Fibroblasts Iron metabolism Iron-binding sites Liver parenchymal cells Transferrin

Abstract According to the hypothesis of Fletcher and Huehns, functional differences exist between both iron-binding sites of transferrin. The site designated A should mainly be involved in the delivery of iron to erythroid cells, whereas site B should donate its iron preferentially to cells involved in the absorption and storage of iron. In the present study this hypothesis could be confirmed by *in vitro* experiments with various cell types. Iron transferrin preincubated with rat bone marrow cells donates less iron to rat bone marrow cells, Chinese hamster fibroblasts, human fibroblasts and human lymphoblasts than freshly prepared iron transferrin equal in iron and transferrin concentration. Rat liver parenchymal cells, however take up more iron from preincubated than from freshly prepared iron transferrin. Obviously site A not only donates iron preferentially to erythroid cells but also to (rapidly) dividing nonerythroid cells in culture. From experiments with iron transferrin mixtures in which radioiron was present at low or high iron saturation, it could be concluded that rat bone marrow cells take up iron equally well from monoferric as from diferric transferrin. The observed functional heterogeneity could, therefore, not be ascribed to differences between monoferric and diferric transferrin.

Introduction

The plasma iron-binding protein transferrin binds two iron atoms at binding sites which are very similar by a variety of criteria [1 2] FLETCH-

ER and HUEHNS [12-14] have postulated that the plasma transferrin iron pool is heterogeneous. From *in vitro* studies they presented evidence that iron uptake by the two binding sites is random, while the release of iron to immature erythroid cells occurs preferentially from one site, arbitrarily designated A site. The other site, designated B site, should be involved with delivery of iron to intestinal and iron storage cells. There is an abundant and contradictory literature concerning the validity of the FLETCHER and HUEHNS hypothesis. Varying results were obtained for various species studied. In the rat it has been established by *in vitro* and *in vivo* experiments that the two binding sites fulfill different physiologic roles [4 5 11 17 18 20]. In the rabbit and human reticulocyte system, however HARRIS and ANSEN [21-23] could not detect a difference between both binding sites.

The aim of the present study was to test the Fletcher and Huehns hypothesis by *in vitro* experiments with various cell types. According to the hypothesis a difference should be found between the behavior of transferrin towards erythroid cells and iron storage cells. Therefore, bone marrow cell suspensions as well as liver parenchymal cells were included in this study. Moreover other cell types which are not directly involved in the main iron metabolism also were tested. As FLETCHER and HUEHNS obtained indications that reticulocytes remove iron more rapidly from transferrin molecules carrying two iron atoms than carrying one, various experiments were carried out to check whether this holds true for rat erythroid cells. This became of importance as contradictory results were obtained by *in vivo* and *in vitro* experiments by a number of authors for rat, rabbit and human cells [11, 15 19 22, 23 6, 27].

Materials and Methods

Rat Bone Marrow Cell Suspensions

Bone marrow cells were isolated from male Wistar rats as described previously [39, 40]. The ratio of myeloid to nucleated erythroid cells was found to be approximately 1:14 from differential counts on smears stained with May-Grünwald-Giemsa stains. The final cell suspension in the incubation medium contained 1.5×10^7 nucleated cells/ml.

Rat Reticulocyte-Rich Blood

Reticulocytosis was induced by repeated phlebotomy in male Wistar rats [40]. The packed cell volume of the blood obtained varied from 0.20 to 0.28 l/l and the re-

iculocyte count from 15 to 26%. The incubation mixtures contained 2.5×10^6 cells/ml.

Rat Liver Cell Suspensions

Liver cell suspensions were essentially prepared as described by HAWARD *et al* [24] and BERRY and FRIEND [9], except that the liver was perfused *in vitro*. The perfusion medium consisted of calcium- and glucose free Hanks solution in which 0.05% collagenase was added. The addition of hyaluronidase (0.1 %) did not improve the yield of viable cells in accordance with the results of MUNTJIK HAAS *et al* [30]. The perfusion technique of SCHOLZ [35] was modified in such a way that it became possible to change the perfusion medium without stopping the perfusion itself. Recirculation of the medium was interrupted after 15 min and the liver flushed with enzyme free medium to remove rubbish material. Subsequently the perfusion was continued with freshly prepared enzyme-containing medium for another period of 15 min. This procedure significantly increased the yield of viable cells as found by the trypan blue exclusion test (0.2% trypan blue in phosphate-buffered saline). Thereafter the liver was cut into small pieces in a medium adapted from VAN BR ZOORJEN *et al* [10]. After 10 min of shaking at 37 °C in a water bath, a CaCl_2 solution was added to a final concentration of 1 mM and incubation was continued for 15 min. After filtering the suspension through nylon gauze, parenchymal cells were isolated by centrifugation at 50 g for 2 min the cells were washed and finally suspended in the incubation mixture to a final concentration of 5×10^6 cells/ml.

In some experiments parenchymal and Kupffer cells were prepared from the same liver by treatment of the supernatant obtained after sedimentation of the parenchymal cells (by centrifugation at 32 g for 2 min) with 0.1 % pronase as described by BIZZO and BOMAN [8].

Fibroblast Cell Lines

Monolayer cultures of the following cell lines were used

a 3 a Chinese hamster fibroblastic line derived from DON [42] CRo control human cell line from the skin of a male and 4/6 Ro a human female fibroblast line, carrier of a balanced translocation between the chromosomes 4 and 6. All cell lines were obtained by courtesy of Dr A. P. M. JONGEMA, Department of Genetics (Head: Prof J. BOOTSMA), Erasmus University Rotterdam. The cells were cultured in a minimum essential medium supplemented with iron (II)-sulphate to a final concentration of 1.5 μM . Before use, the monolayers were thoroughly washed with phosphate-buffered saline to remove iron (II)-sulphate, treated with 0.25% trypsin and subsequently harvested by centrifugation at 200 g for 7 min. After resuspension in the incubation medium the cells were counted and approximately 10^4 cells were added to culture dishes for incubation with transferrin.

Human Lymphoid C II Cultures

Suspensions of FJHR 1 cells, derived from a malign lymphoma [44] were a generous gift of Dr ZORRILLA MALLAOS (Department of Hematology Academic Hospital Rotterdam-Dijkzigt, Rotterdam). After centrifugation at 200 g for 7 min the cells

were resuspended in the incubation medium to final concentration of 5×10^4 cells/ml.

Iron Transferrin Mixtures

Iron-free transferrin was prepared by dialyzing twice against 0.02 M disodium EDTA in 0.05 M sodium acetate buffer (pH 5.0). EDTA was subsequently removed by repeated dialysis against bidistilled water whereafter transferrin was lyophilized in small portions. Each portion was solubilized in phosphate-buffered saline so as to obtain a concentration of 3 mg/ml as measured by the absorbance at 280 nm with $\epsilon_{280}^{1\%} = 11.3$. To 10 ml of transferrin solution (^{55}Fe) ferric citrate with specific radioactivity of approximately 0.2 $\mu\text{Ci}/\mu\text{mol}$ of iron was added, and the mixtures were incubated at 37 °C for 1 h. Electrophoresis on cellulose acetate indicated that at least 90% of the ^{55}Fe was transferrin-bound [39]. Where indicated iron transferrin was prepared with $^{55}\text{Fe}(\text{III})$ -chloride in a similar way. In some experiments at first nonradioactive iron (added as ferric citrate) was bound to transferrin, followed by the binding of radioactive iron. In some other experiments the reverse sequence was followed. Iron saturations are given in the legends to the figures. Iodination of transferrin with ^{125}I was carried out as described earlier [40].

Incubation Procedures

Incubation was carried out in minimum essential medium in an atmosphere of 9% $\text{CO}_2/91\%$ air as described previously [37-40]. The amount of labeled transferrin and its iron saturation is given in the legends to the figures. After incubation the cells were washed 5 times with ice-cold Hank's balanced salt solution at 0-4 °C. The radioactivity of the cells was estimated in a Packard scintillation spectrometer, Model 5320 (Packard Instruments, Downers Grove, Ill.). In some experiments the radioactivity of iron-59 incorporated into haem was determined [40].

Precubation was carried out with reticulocyte-rich blood or bone marrow cells in the presence of ^{55}Fe bound to ^{125}I -transferrin under the same conditions. After 4 h of incubation the cells were separated from the supernatant by centrifugation and the amount and saturation of transferrin was calculated from the ^{55}Fe and ^{125}I radioactivity. In the control mixture ^{125}I -transferrin, saturated to the calculated degree with ^{55}Fe , was added to fresh medium to obtain the same concentration. Various cells were, thereafter resuspended in the precubated and freshly prepared medium and subsequently incubated as described above.

Media/Reagents

Rat transferrin was isolated and purified as described previously [37]. Human transferrin was purchased from Kabi, Stockholm, Sweden. ^{55}Fe was obtained as sterile ferric citrate or as ferric chloride, and ^{125}I as sodium iodide, carrier-free and free from reducing agents, from the Radiochemical Centre, Amersham, Bucks, UK. Collagenase (type I), hyaluronidase (type II) and bovine serum albumin (fraction V) were obtained from the Sigma Chemical Co. (St. Louis, Mo.), pronase (B grade) from Calbiochem (San Diego, Calif.), and trypan blue from BDH Chemicals (Poole, Dorset, UK). Cell counting was performed with a hemocytometer or with an electronic cell counter (MCC-1002 B, Tom Electric, Kobe, Japan).

Results

Functional Heterogeneity of Iron-Binding Sites

In preliminary experiments, iron uptake by rat liver parenchymal cells was studied with both $^{59}\text{Fe}(\text{III})$ -citrate and ^{59}Fe bound to rat transferrin, as liver cell suspensions were reported to take up inorganic iron complexes rather efficiently [6-43]. Iron bound to transferrin appeared to be less available for liver cells than ferric citrate (fig. 1) in contrast to earlier results with rat bone marrow cells [39]. This phenomenon could not be ascribed to the presence of small amounts of Kupffer cells present in the preparation of parenchymal cells as a comparable difference between the iron uptake from ferric citrate and iron transferrin was found with isolated Kupffer cells, although the total amount of iron taken up by these cells was significantly less than by parenchymal cells for both ferric citrate and iron transferrin. Whether parenchymal cells were incubated with ferric citrate or with iron transferrin, in both cases only 1-2% of the iron-59

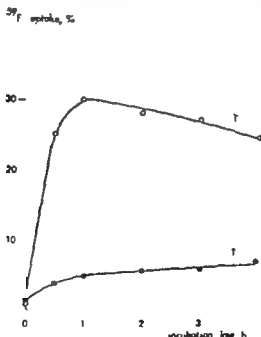


Fig. 1 Iron uptake by rat liver parenchymal cells. Cells were incubated with $0.3 \mu\text{M}$ $^{59}\text{Fe}(\text{III})$ -citrate (-Tr) or with $0.3 \mu\text{M}$ ^{59}Fe bound to rat transferrin (+Tr). Transferrin saturation was 40%. Uptake is expressed as percentage of the amount of ^{59}Fe added.

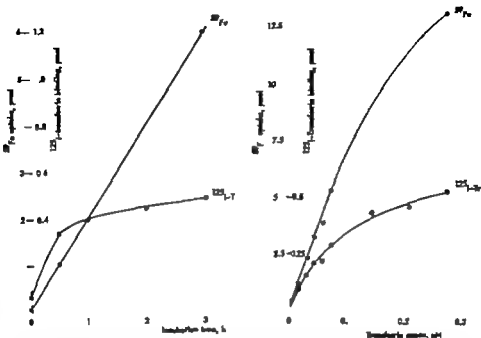


Fig. 2. Iron uptake and transferrin binding by Chinese hamster fibroblasts (a3). Human ^{125}I -transferrin, saturated to 45% with ^{59}Fe , was used. *a* Effect of the time of incubation. Transferrin concentration was 0.2 μM . *b* Effect of the transferrin concentration. Incubation time was 2 h. Results are expressed as pmol of ^{59}Fe taken up and ^{125}I -transferrin bound by the cells per incubation mixture containing 10^6 cells.

● = ^{59}Fe uptake; ○ = ^{125}I -transferrin binding.

taken up by the cells was found to be incorporated into haem. Probably a large part of the iron taken up is incorporated into ferritin [25]. In figure 2 the results with Chinese hamster fibroblasts are shown. Iron uptake increases linearly for at least 3 h, whereas human ^{125}I -transferrin binding reaches an equilibrium within 30 min, in agreement with the results of Messmer [29]. The transferrin binding and iron uptake at varying iron-transferrin concentrations (fig. 2b) is also very similar to the results obtained by others [29]. Only very small parts (less than 2%) of the iron-59 taken up by the cells could be recovered into the haemin fraction. Comparable results as obtained with Chinese hamster fibroblasts were found with various types of human fibroblasts and with human lymphoblasts.

Results

Functional Heterogeneity of Iron Binding Sites

In preliminary experiments, iron uptake by rat liver parenchymal cells was studied with both $^{59}\text{Fe}(\text{III})$ -citrate and ^{59}Fe bound to rat transferrin, as liver cell suspensions were reported to take up inorganic iron complexes rather efficiently [6-43]. Iron bound to transferrin appeared to be less available for liver cells than ferric citrate (fig. 1) in contrast to earlier results with rat bone marrow cells [39]. This phenomenon could not be ascribed to the presence of small amounts of Kupffer cells present in the preparation of parenchymal cells, as a comparable difference between the iron uptake from ferric citrate and iron transferrin was found with isolated Kupffer cells, although the total amount of iron taken up by these cells was significantly less than by parenchymal cells for both ferric citrate and iron transferrin. Whether parenchymal cells were incubated with ferric citrate or with iron transferrin, in both cases only 1-2% of the iron ^{59}Fe

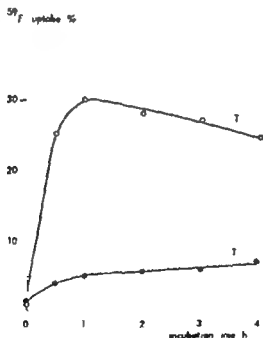


Fig 1 Iron uptake by rat liver parenchymal cells. Cells were incubated with $0.3 \mu\text{M}$ $^{59}\text{Fe}(\text{III})$ -citrate (-Tr) or with $0.3 \mu\text{M}$ ^{59}Fe bound to rat transferrin (+Tr). Transferrin saturation was 40%. Uptake is expressed as percentage of the amount of ^{59}Fe added.

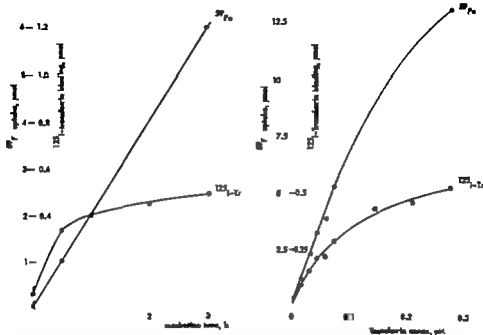


Fig. 2. Iron uptake and transferrin binding by Chinese hamster fibroblasts (a5). Human ^{59}Fe -transferrin, saturated to 49% with ^{59}Fe , was used. Effect of the time of incubation. Transferrin concentration was 0.2 μM . b Effect of the transferrin concentration. Incubation time was 2 h. Results are expressed as pmol of ^{59}Fe taken up and ^{125}I -transferrin bound by the cells per incubation mixture containing 10^4 cells.

● = ^{59}Fe uptake ○ = ^{125}I -transferrin binding.

taken up by the cells was found to be incorporated into haem. Probably a large part of the iron taken up is incorporated into ferritin [25]. In figure 2 the results with Chinese hamster fibroblasts are shown. Iron uptake increases linearly for at least 3 h, whereas human ^{125}I -transferrin binding reaches an equilibrium within 30 min, in agreement with the results of MESSNER [29]. The transferrin binding and iron uptake at varying iron-transferrin concentrations (fig. 2b) is also very similar to the results obtained by others [29]. Only very small parts (less than 2%) of the iron-59 taken up by the cells could be recovered into the haemin fraction. Comparable results as obtained with Chinese hamster fibroblasts were found with various types of human fibroblasts and with human lymphoblasts.

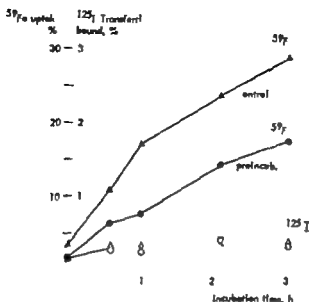


Fig 3 Effect of preincubation on iron uptake and transferrin binding by rat bone marrow cells. Preincubation was carried out with reticulocyte-rich blood. Rat ^{125}I -transferrin (saturated to 80% with ^{59}Fe) concentration was $1.0\text{ }\mu\text{M}$. After 4 h of incubation the saturation was decreased to 50%. Rat bone marrow cells were suspended in this preincubated medium as well as in freshly prepared medium containing the same concentration of ^{125}I transferrin saturated to 50% with ^{59}Fe . Incubation was performed during the times indicated. Results are expressed as percent of the amounts of ^{59}Fe and ^{125}I present in the mixtures. Closed symbols indicate ^{59}Fe uptake and open symbols ^{125}I -transferrin binding.

To study the functional heterogeneity of iron binding sites, at first the experiment originally carried out by FLETCHER and HUEBNS [12] in which transferrin iron preincubated in the presence of reticulocytes appeared to transfer iron less efficiently to reticulocytes than freshly prepared iron transferrin was repeated with rat bone marrow cells. Essentially the same results were found (fig 3). No differences in ^{125}I transferrin binding could be observed, suggesting that the binding of transferrin to the cell receptors is not influenced by preincubation. In figure 4 the results of similar experiments with Chinese hamster cells are presented. Transferrin preincubated with rat bone marrow cells also appeared to donate iron less efficiently to these cells than not preincubated transferrin. The results with various types of human fibroblasts and with human lymphoblasts were essentially the same (not shown).

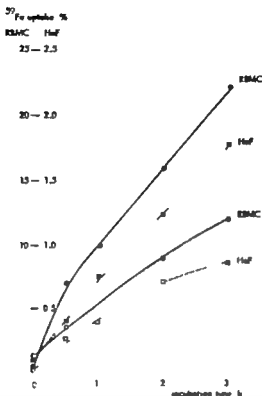


Fig 4 Effect of preincubation on iron uptake by rat bone marrow cells (RBMC) and Chinese hamster fibroblasts (a3) (HaF). Preincubation was carried out with rat bone marrow cells in the presence of 0.3 μ M 59 I-transferrin saturated to 80% with 59 Fe. Control mixtures were prepared as described under 'Materials and Methods'. Results are expressed as percent 59 F taken up by the cells. Open symbols represent iron uptake from preincubated transferrin, closed symbols from control mixtures. Preincubation decreased iron saturation of human transferrin from 80 to 54%. Preincubation did not have any effect on the 59 I-transferrin binding. Results are, therefore, not shown.

By contrast, the opposite effect was found when the second incubation was carried out in the presence of rat liver cell suspensions. In table I the results of two experiments are presented. Preincubated transferrin donates its iron to liver cells better than freshly prepared iron transferrin. As a control, in the same experiments rat bone marrow cells were incubated

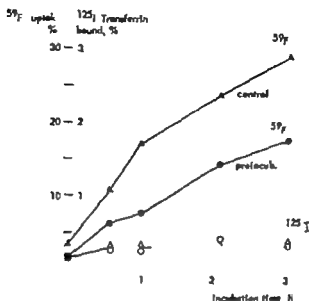


Fig 3 Effect of preincubation on iron uptake and transferrin binding by rat bone marrow cells. Preincubation was carried out with reticulocyte-rich blood. Rat ^{125}I -transferrin (saturated to 80% with ^{59}Fe) concentration was $1.0\ \mu\text{M}$. After 4 h of incubation the saturation was decreased to 50%. Rat bone marrow cells were suspended in this preincubated medium as well as in freshly prepared medium containing the same concentration of ^{125}I -transferrin saturated to 50% with ^{59}Fe . Incubation was performed during the times indicated. Results are expressed as percent of the amounts of ^{59}Fe and ^{125}I present in the mixtures. Closed symbols indicate ^{59}Fe uptake and open symbols ^{125}I -transferrin binding.

To study the functional heterogeneity of iron-binding sites, at first the experiment originally carried out by FLETCHER and HUEBNS [12] in which transferrin iron preincubated in the presence of reticulocytes appeared to transfer iron less efficiently to reticulocytes than freshly prepared iron transferrin was repeated with rat bone marrow cells. Essentially the same results were found (fig. 3). No differences in ^{125}I -transferrin binding could be observed, suggesting that the binding of transferrin to the cell receptors is not influenced by preincubation. In figure 4 the results of similar experiments with Chinese hamster cells are presented. Transferrin preincubated with rat bone marrow cells also appeared to donate iron less efficiently to these cells than not preincubated transferrin. The results with various types of human fibroblasts and with human lymphoblasts were essentially the same (not shown).

From the cells studied only rat liver cells appeared to take up iron better from preincubated iron transferrin than from freshly prepared mixtures.

The Effect of Iron Transferrin Saturation

The experiments described above were carried out with transferrin initially saturated to 80% with iron. After preincubation the saturation was reduced to 40–60%. It has been reported [26] that differences in reticulocyte iron uptake from preincubated and not-preincubated iron transferrin depends on the saturation degree of transferrin. To study whether this holds true also for bone marrow cells, the following experiment was carried out. Rat bone marrow cells were preincubated with ^{59}Fe -transferrin saturated with iron-59 for 79 and 44 %, respectively. After 3 h of incubation with bone marrow cells iron saturation was reduced to 33 and 18%. Subsequently freshly isolated rat bone marrow cells were added to these samples and the iron-59 uptake compared with control mixtures containing 33 and 18% iron-saturated transferrin. Iron uptake was found to be independent on the iron saturation of transferrin, whereas the Fletcher Huehns effect was the same for both saturation degrees (table III).

According to various authors the Fletcher Huehns effect may be explained, at least partially by the finding that diferric transferrin is a better iron donor for erythroid cells than monoferric transferrin [11 19 26]. To study this, nonlabeled iron (III)-citrate was added to six samples of transferrin to obtain saturation degrees varying from 0 to 86%. After iron binding, various amounts of $^{59}\text{Fe(III)}$ -citrate were added and the iron transferrin samples so obtained incubated with rat bone marrow cells for 3.5 h. Iron uptake was found almost linearly with increasing saturation (fig. 5a). Moreover a series of more or less parallel lines were obtained,

Table III. Effect of initial iron saturation of transferrin on the iron uptake by rat bone marrow cells after preincubation

Iron saturation, % before preincubation	^{59}Fe uptake, %		
	after preincubation	preincubated	control
79	33	40	47
44	18	41	48

Results are expressed as percent of iron- 59 taken up by the cells. Transferrin concentration was 0.3 μM and the incubation time 3 h.

Table I Effect of preincubation on iron uptake by rat liver parenchymal and bone marrow cells

Experiment No.	Cells	⁵⁵ Fe uptake, pmol		Ratio I II
		I	II	
1	Parenchymal	8.3	3.2	2.60
	bone marrow	115	186	0.62
2	parenchymal	34	26	1.31
	bone marrow	69	89	0.78

Preincubation was carried out with rat bone marrow cells in the presence of 0.25 μ M rat transferrin saturated to 80% with ⁵⁵Fe. After 5 h of incubation the saturation was decreased to 63% (exp. 1) and 58% (exp. 2). Rat liver parenchymal and bone marrow cells were suspended in preincubated medium (I) and in freshly prepared medium with the same saturation degree of transferrin (II). Incubation was carried out for 2 h.

Table II Effect of preincubation on iron uptake by various cell types as compared with rat bone marrow cells

Cell type	Species of transferrin	Saturation of transferrin after preincubation, %	Ratio ⁵⁵ Fe uptake	Ratio for rat bone marrow cells
Rat reticulocytes	rat	50	0.70	0.60
Rat spleen cells	rat	45	0.55	0.50
Rat liver parenchymal cells	rat	55	1.95	0.60
Chinese hamster fibroblasts	human	83	0.51	0.51
Human fibroblasts	human	45	0.33	0.37
Human lymphoblasts	rat	44	0.68	0.42

Experiments were carried out as described in the legend to table I. Ratios of ⁵⁵Fe uptake from preincubated ⁵⁵Fe-[¹²⁵I]transferrin to not-preincubated transferrin are given. Initial saturation degree was 80%. Incubation was performed for 2 h. Mean of triplicates are given.

ed with the same ⁵⁵Fe transferrin mixtures. The ratios of ⁵⁵Fe uptake from preincubated and not preincubated ⁵⁵Fe-transferrin for liver parenchymal and bone marrow cells appeared to vary from one experiment to another probably due to the efficiency of preincubation as lower ratios for parenchymal cells coincided with higher ratios for bone marrow cells. In table II the results of experiments with various types of cells are summarized.

iron (fig. 5b, broken lines), the total (labeled and nonlabeled) amount of iron taken up by the cells did not differ whether nonlabeled iron was added at 15, 36 or 70% saturation with iron-59 (fig. 5b, solid lines). These results also strongly suggest that diferric transferrin does not donate iron to these cells at higher rates than monoferric transferrin. As there are some objections against the use of iron citrate to bind iron to transferrin [3, 16, 20] similar experiments were performed in which ferric-citrate was replaced by ferric-chloride. Essentially the same results could be recorded. Moreover if the presence of citrate, under the conditions applied, should induce an exchange of iron bound to binding sites of transferrin, no Fletcher Huehns effect could be expected. This effect was, however clearly demonstrated.

Discussion

According to the hypothesis of Fletcher and Huehns, the iron-binding sites of transferrin are physiological heterogeneous. Site A should deliver iron to erythroid cells, whereas site B should be involved in delivering iron to the liver and intestine. In the rat, *in vivo* experiments are in support of this hypothesis [4, 5, 11, 18, 31]. *In vitro* experiments with rat cells were only carried out with reticulocytes [17, 18] with the exception of a recent study [7] in which liver cell cultures obtained from a cell line derived from parenchymal cells from rat adult liver were investigated. The latter results did not agree with the Fletcher-Huehns hypothesis and were contradictory to the results of *in vivo* experiments in the rat. The aim of the present study was to examine the validity of the Fletcher-Huehns hypothesis using rat erythroid cells as well as a number of rat, human and hamster cell types.

⁵⁹Fe-transferrin which had been preincubated with rat bone marrow cells delivered significantly less iron-59 to bone marrow cells than fresh preparations of ⁵⁹Fe-transferrin equal in iron and transferrin concentration. Apparently rat bone marrow cells preferentially take up iron from one iron-binding site, presumably the site designated A by Fletcher and Huehns. In this respect the results with bone marrow cells are identical with those with rat reticulocytes [17, 20, 33]. *In vitro* the A site also delivers iron preferentially to human and Chinese hamster fibroblasts and human lymphoblasts (table II). In contrast, the other site (B site) was found to donate its iron preferentially to rat liver parenchymal cells as iron transferrin preincubated with bone marrow cells is a better iron do-

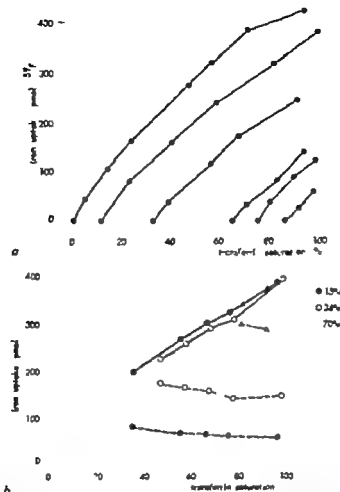


Fig 5 Effect of transferrin saturation on iron uptake by bone marrow cells. *a* To human apotransferrin and transferrin saturated with nonlabeled iron to 10, 32, 64 and 86%, varying amounts of ^{59}Fe (as ferric citrate) were bound. The mixtures (final transferrin concentration $1 \mu\text{M}$) were incubated with rat bone marrow cells for 3.5 h and the amount of ^{59}Fe taken up by the cells, expressed as pmol per incubation mixture, determined. *b* To human ^{59}Fe transferrin with saturation degrees of 15, 36 and 70%, varying amounts of nonlabeled iron (as ferric citrate) were bound. Iron uptake by bone marrow cells was determined after 3.5 h. Broken lines ^{59}Fe uptake (pmol) solid lines = total iron uptake (pmol).

indicating that iron 59 bound at low saturation is taken up by the cells at the same rate as iron bound at high saturation. In the reverse experiment first iron 59 was bound to transferrin followed by nonlabeled iron. Although the amount of iron-59 taken up by rat bone marrow cells decreased somewhat upon increasing the saturation degree with nonlabeled

iron (fig. 5b, broken lines) the total (labeled and nonlabeled) amount of iron taken up by the cells did not differ whether nonlabeled iron was added at 15, 36 or 70% saturation with iron-59 (fig. 5b, solid lines). These results also strongly suggest that diferric transferrin does not donate iron to these cells at higher rates than monoferric transferrin. As there are some objections against the use of iron citrate to bind iron to transferrin [3, 16, 20] similar experiments were performed in which ferric-citrate was replaced by ferric-chloride. Essentially the same results could be recorded. Moreover if the presence of citrate, under the conditions applied, should induce an exchange of iron bound to binding sites of transferrin, no Fletcher Huehns effect could be expected. This effect was, however clearly demonstrated.

Discussion

According to the hypothesis of Fletcher and Huehns, the iron-binding sites of transferrin are physiological heterogeneous. Site A should deliver iron to erythroid cells, whereas site B should be involved in delivering iron to the liver and intestine. In the rat, *in vivo* experiments are in support of this hypothesis [4, 5, 11, 18, 31]. *In vitro* experiments with rat cells were only carried out with reticulocytes [17, 18] with the exception of a recent study [7] in which liver cell cultures obtained from a cell line derived from parenchymal cells from rat adult liver were investigated. The latter results did not agree with the Fletcher Huehns hypothesis and were contradictory to the results of *in vivo* experiments in the rat. The aim of the present study was to examine the validity of the Fletcher Huehns hypothesis using rat erythroid cells as well as a number of rat, human and hamster cell types.

^{59}Fe -transferrin which had been preincubated with rat bone marrow cells delivered significantly less iron 59 to bone marrow cells than fresh preparations of ^{59}Fe -transferrin equal in iron and transferrin concentration. Apparently rat bone marrow cells preferentially take up iron from one iron-binding site, presumably the site designated A by Fletcher and Huehns. In this respect the results with bone marrow cells are identical with those with rat reticulocytes [17, 20, 33]. *In vitro* the A site also delivers iron preferentially to human and Chinese hamster fibroblasts and human lymphoblasts (table II). In contrast, the other site (B site) was found to donate its iron preferentially to rat liver parenchymal cells as iron transferrin preincubated with bone marrow cells is a better iron do-

nor for these cells than a freshly prepared iron transferrin mixture (table I). These results are in support with the Fletcher Huehns hypothesis and with *in vivo* experiments in rats [4-18]. Concerning other animal species HARRIS and AISEN [21-23] raised some questions as they did observe a difference between the iron binding sites by studying human transferrin with rabbit reticulocytes but not in the homologous rabbit and human systems. Whether this reflects a real difference between various species or methodological differences, remains to be solved. In this context it seems relevant to note that significant differences exist between human and rat reticulocytes in their capability to take up iron and to synthesize haem as could be established from iron 59 uptake studies and from assays of the enzyme haem synthetase (ferro-chelatase) [41].

From experiments in which rat serum transferrin was preincubated with rat reticulocytes and thereafter injected into rats, AWAI *et al* [4] found a preferential uptake of iron from the A site by bone marrow and from the B site by liver and small intestine. Our results with liver cell suspensions are in agreement with their *in vivo* experiments, but contradictory to the results of *in vitro* experiments of BEAMISH *et al* [7]. These authors found no differences between rat liver cell cultures (obtained from a cell line derived from parenchymal cells from rat adult liver), rat embryonic cell cultures and rat reticulocytes in their preference for one of the iron-binding sites of transferrin. Possibly the growth in culture for many generations did alter membrane characteristics involved in the selective iron uptake by the cells, characteristics which are not eliminated by the isolation procedure of fresh liver cells as applied in our studies.

The results of the experiments in which radioliron was bound to transferrin saturated with iron 56 to various degrees (fig 5) strongly suggest that the rate of iron uptake by rat bone marrow cells is not influenced by the degree of iron saturation of transferrin. Moreover under the same conditions monoferric and diferric transferrin appeared to have very similar affinities for rat bone marrow cells [38]. Considerable disagreement exists concerning the iron uptake from monoferric and diferric transferrin by reticulocytes *in vitro* [22, 23-26] and the rate at which iron from monoferric and diferric transferrin is cleared from the plasma after intravenous injection of transferrin into humans or rats [11, 15, 19, 27]. It seems, therefore, unlikely that in general differences between monoferric and diferric transferrin play a functional role in the process of iron uptake by erythroid cells. The absence of any difference between monoferric and diferric transferrin as found in the present study could not be ascribed to

the presence of citrate, which appears to promote a redistribution of iron between both iron-binding sites after prolonged incubation [3, 16, 20] as the same results were obtained when ferric chloride was used to label transferrin. Moreover a redistribution of iron should give no differences between both iron-binding sites in their delivery of iron to various cell types. Such differences could be clearly demonstrated (fig. 3, 4, table I, II).

Finally it appears desired to make some remarks on the iron uptake by fibroblasts and lymphoblasts in culture. It is rather surprising that fibroblasts in cell cultures take up such large amounts of iron, as *in vivo* iron turnover in such tissues is so small that ferrokinetic measurements cannot detect it [2]. Similar observations are made for lymphoid cells, as lymphoblasts in culture [this paper] and phytohemagglutinin-stimulated lymphocytes [32] take up significant amounts of iron, whereas iron uptake *in vivo* by lymphoid cells could not be observed [36]. Probably iron requirement is related to the rate at which cells divide. This suggestion is supported by the finding that iron transferrin stimulates the uptake of thymidine and uridine and the synthesis of DNA and RNA in phytohemagglutinin-stimulated lymphocytes but not in nonstimulated lymphocytes [32]. It has also to be noted that the ribonucleotide-reductase enzyme complex contains iron [28] and that blocking the entry of iron into HeLa cells by desferrioxamine inhibits DNA synthesis [34].

Acknowledgement

The authors are indebted to Dr. A. P. M. JONSSMA (Department of Genetics) for gifts of cell cultures of fibroblasts and many helpful suggestions. Miss EVELINE VAN 'T HULL (Department of Pathological Anatomy) and Dr. H. ZONDELA-MALLIEUX (Department of Hematology) are gratefully acknowledged for performing differential counts on rat bone marrow and for gifts of suspensions of P3HR-1 lymphoblastoid cells, respectively.

References

1. ARSEN, P. and BROWN, E. B., Structure and function of transferrin, in *Brown Progress in hematology* vol. 9, pp. 25-56 (Grune & Stratton, New York 1975).
2. ARSEN, P. and BROWN, E. B., The iron-binding function of transferrin in iron metabolism, in *Afrschoer and Jaffe Seminars in hematology* vol. 14, pp. 31-53 (Grune & Stratton, New York 1977).
3. ARSEN, P. and LEBMAN, A., Citrate-mediated exchange of Fe^{3+} among transferrin molecules. *Biochem. biophys. Res. Commun.* 52, 220-226 (1966).

- 4 AWAL, M. CHIPMAN, B., and BROWN, E. B. *In vivo* evidence for the functional heterogeneity of transferrin-bound iron. I. Studies in normal rats. *J. Lab. clin. Med.* 85 769-784 (1975)
- 5 AWAL, M. CHIPMAN, B. and BROWN, E. B. *In vivo* evidence for the functional heterogeneity of transferrin-bound iron. II. Studies in pregnant rats. *J. Lab. clin. Med.* 85 785-796 (1975)
- 6 BASS, R. and SALTMAN, P.. The accumulation of iron by rat liver cell suspensions. *Exptl Cell Res.* 18 560-572 (1959)
- 7 BEAMISH, M. R. KEAY, L., OKUDA, T. and BROWN, E. B. Uptake of transferrin bound iron by rat cells in tissue culture. *Br. J. Haemat.* 31 479-491 (1975).
- 8 BERG, T. and BOMAN, D. Distribution of lysosomal enzymes between parenchymal and Kupffer cells of rat liver. *Biochim. biophys. Acta* 321 585-596 (1973).
- 9 BERRY, M. N. and FRIEND, D. S.. High-yield preparation of isolated rat liver parenchymal cells. A biochemical and fine structural study. *J. Cell Biol.* 43 506-520 (1969)
- 10 BEZOOGEN, C. F. A. VAN NOORD, M. J. VAN and KROOK, D. L.. The viability of parenchymal liver cells isolated from young and old rats. *Mech. Age Dev.* 3 107-119 (1974)
- 11 BROWN, E. B., OKADA, S., AWAL, M. and CHIPMAN, B. *In vivo* evidence for the functional heterogeneity of transferrin-bound iron. III. Studies of transferrin at high and low iron saturation. *J. Lab. clin. Med.* 86 576-583 (1975).
- 12 FLETCHER, J. and HUIZINGA, E. R.. Significance of the binding of iron by transferrin. *Nature, Lond.* 215 584-586 (1967).
- 13 FLETCHER, J. and HUIZINGA, E. R.. Function of transferrin. *Nature, Lond.* 218 1211-1214 (1968).
- 14 FLETCHER, J.. Variation in the availability of transferrin-bound iron for uptake by immature red cells. *Clin. Sci.* 37 273-297 (1969)
- 15 FLETCHER, J.. The plasma clearance and liver uptake of iron from transferrin of low and high iron saturation. *Clin. Sci.* 41 395-400 (1971).
- 16 GANZONI, A. M., BAVIERA, B., and HAHN, D. Transferrin. Intermolecular iron exchange. *Experientia* 30 32 (1974).
- 17 GANZONI, A. M., HAHN, D. and SPÄTZ, B.. Plasma iron transport. Absence of a uniform system. *Blut* 4 269-273 (1972)
- 18 HAHN, D. Functional behaviour of transferrin. *Eur. J. Biochem.* 34 311-316 (1973)
- 19 HAHN, D., BAVIERA, B. and GANZONI, A. M. Functional heterogeneity of the transport iron compartment. I. *In vivo* radiolabelled iron clearance from high and low saturated transferrin. *Acta haemat.* 53 285-291 (1975).
- 20 HAHN, D. and GANZONI, A. M. Functional heterogeneity of the transport iron compartment. II. *In vivo* differences between transferrin iron binding sites, and *in vitro* interbinding site iron exchange. *Acta haemat.* 53 321-328 (1975)
- 21 HARRIS, D. C. Functional equivalence of iron bound to human transferrin at low pH or high pH. *Biochim. biophys. Acta* 496 563-565 (1977)
- 22 HARRIS, D. C. and ARDEN, P. Iron-donating properties of transferrin. *Biochemistry* 14 262-268 (1975).

- 23 HARRIS, H. C. and ARSEN, P. Functional equivalence of the two iron-binding sites of human transferrin. *Nature, Lond.* 257 821-823 (1975).
- 24 HAWARD, R. B., CHRISTENSEN, A. K., GIBBS, P. A., and PERCH, L. A. The enzymatic preparation of isolated intact parenchymal cells from rat liver. *J. Cell Biol.* 35 675-684 (1967).
- 25 KERTZ, B. K., VAN PINDENBURG, A. M. C. M., EDE, H. G. VAN, and LAURIE, B. The incorporation of ^{59}Fe , ^3H leucine and ^{32}P inorganic phosphate in ferritin during the perfusion of isolated rat livers. *Biochim. biophys. Acta* 273 243-247 (1972).
- 26 LANE, R. S. Iron uptake by rabbit reticulocytes. *Br. J. Haemat.* 24 343-353 (1973).
- 27 LANE, R. S. and FRICK, C. A. The *in vivo* plasma clearance of iron from transferrin of low and high iron saturation. *Clin. Sci.* 58 783-793 (1970).
- 28 LARSSON, A. Ribonucleotide reductase from regenerating rat liver. *Eur. J. Biochem.* 11 113-121 (1969).
- 29 MENDLER, T. O. A comparison of the interaction of Chinese hamster fibroblasts with human and bovine transferrins, and conalbumin (chick-egg transferrin). *Biochim. biophys. Acta* 320 663-670 (1973).
- 30 MINNICH KAS, A. C., BRZO, T., SEGLER, P. O. and SELBY, R. Mass isolation and culture of rat Kupffer cells. *J. exp. Med.* 141 1-10 (1975).
- 31 OKADA, S., CHIDMAN, B., and BROWN, E. B. *In vivo* evidence for the functional heterogeneity of transferrin-bound iron. IV Selective uptake by erythroid precursors of radioiron from portal vein plasma transferrin during intestinal iron absorption. *J. Lab. clin. Med.* 89 51-64 (1977).
- 32 PHELPS, J. L. and AZARI, P. Effect of iron transferrin on nucleic acid synthesis in phytohemagglutinin-stimulated human lymphocytes. *Cell. Immunol.* 15 94-99 (1975).
- 33 PERACOTTO, J. V. and ZAPOLSKY, E. J. Fractional heterogeneity and pH-dependent dissociation properties of human transferrin. *Biochim. biophys. Acta* 428 766-771 (1976).
- 34 ROSSIGNOL, E., FANT, J. and NORTON, W. Intracellular iron-binding macromolecules in HeLa cells. *Proc. natn. Acad. Sci. USA* 69 3708-3712 (1972).
- 35 SCHOLZ, R. Untersuchungen zur Redoxkompartimentierung bei der hämoglobinfreien perfundierten Rattenleber. In STÄHL and SCHOLZ Stoffwechsel der isoliert perfundierten Leber 3. Konf. Ges. Biologische Chemie, Oestrich/Rheingau 1967 (Springer, Berlin 1968).
- 36 TARRITT, R. O. A study of erythropoiesis in the rat. *Exptl Cell Res.* 48 473-483 (1967).
- 37 VERHOEF, N. J. and EDE, H. G. VAN. Isolation, characterization and function of cord-blood transferrin. *Clin. Sci. Mol. Med.* 48 335-340 (1975).
- 38 VERHOEF, N. J., KESTER, H. C. M., NOORDHOOF, P. J. and LAURIE, B. *In vitro* studies on the interaction between transferrin and rat bone marrow cells (in press).
- 39 VERHOEF, N. J., KARMERS, J. H. W. and LAURIE, B. The effect of heterologous transferrin on the uptake of iron and haem synthesis by bone marrow cells. *Biochim. biophys. Acta* 304 114-122 (1973).

- 4 AWAI, M., CHIPMAN, B. and BROWN, E. B. *In vivo* evidence for the functional heterogeneity of transferrin-bound iron. I. Studies in normal rats. *J. Lab. clin. Med.* 85 769-784 (1975).
- 5 AWAI, M., CHIPMAN, B., and BROWN, E. B. *In vivo* evidence for the functional heterogeneity of transferrin-bound iron. II. Studies in pregnant rats. *J. Lab. clin. Med.* 85 785-796 (1975).
- 6 BASS, R. and SALTMAN, P. The accumulation of iron by rat liver cell suspensions. *Expl. Cell. Res.* 18 560-572 (1959).
- 7 BRAMERT, M. R., KEAY, L., OKIGAKI, T., and BROWN, E. B. Uptake of transferrin-bound iron by rat cells in tissue culture. *Br. J. Haemat.* 31 479-491 (1975).
- 8 BERG, T. and BOMAN, D. Distribution of lysosomal enzymes between parenchymal and Kupffer cells of rat liver. *Biochim. biophys. Acta* 321 585-596 (1973).
- 9 BERRY, M. N. and FRIEND, D. S. High-yield preparation of isolated rat liver parenchymal cells. A biochemical and fine structural study. *J. Cell Biol.* 43 506-520 (1969).
- 10 BEZOOUDEN, C. F. A. VAN NOORD, M. J. VAN and KNOOK, D. L. The viability of parenchymal liver cells isolated from young and old rats. *Mech. Age Dev.* 3 107-119 (1974).
- 11 BROWN, E. B., OKADA, S., AWAI, M. and CHIPMAN, B. *In vivo* evidence for the functional heterogeneity of transferrin-bound iron. III. Studies of transferrin at high and low iron saturation. *J. Lab. clin. Med.* 86 576-585 (1975).
- 12 FLETCHER, J. and HUENES, E. R. Significance of the binding of iron by transferrin. *Nature, Lond.* 215 584-586 (1967).
- 13 FLETCHER, J. and HUENES, R. Function of transferrin. *Nature, Lond.* 218 1211-1214 (1968).
- 14 FLETCHER, J. Variation in the availability of transferrin-bound iron for uptake by immature red cells. *Clin. Sci.* 37 273-297 (1969).
- 15 FLETCHER, J. The plasma clearance and liver uptake of iron from transferrin of low and high iron saturation. *Clin. Sci.* 41 395-402 (1971).
- 16 GANZONI, A. M., BAVIERA, B. and HAHN, D. Transferrin. Intermolecular iron exchange. *Experientia* 30 32 (1974).
- 17 GANZONI, A. M., HAHN, D. and SPÄTZ, B. Plasma iron transport. Absence of a uniform system. *Blut* 24 269-273 (1972).
- 18 HAHN, D. Functional behaviour of transferrin. *Eur. J. Biochem.* 34 311-316 (1973).
- 19 HAHN, D., BAVIERA, B. and GANZONI, A. M. Functional heterogeneity of the transport iron compartment. I. *In vivo* radioiron clearance from high and low saturated transferrin. *Acta haemat.* 53 285-291 (1975).
- 20 HAHN, D. and GANZONI, A. M. Functional heterogeneity of the transport iron compartment. II. *In vivo* differences between transferrin iron binding sites, and *in vivo* interbinding site iron exchange. *Acta haemat.* 53 321-328 (1975).
- 21 HARRIS, D. C. Functional equivalence of iron bound to human transferrin at low pH or high pH. *Biochim. biophys. Acta* 496 563-565 (1977).
- 22 HARRIS, D. C. and ANKEN, P. Iron-donating properties of transferrin. *Biochemistry* 14 262-268 (1975).

- 23 HARRIS, D. C. and ARSEN, F. Functional equivalence of the two iron-binding sites of human transferrin. *Nature, Lond.* 257 821-823 (1975).
- 24 HAWARD, R. B., OGGSTEDTSEN, A. K., GUNES, F. A., and PERCH, L. A. The enzymatic preparation of isolated intact parenchymal cells from rat liver. *J. Cell Biol.* 33, 675-684 (1967).
- 25 KREUZ, B. K. VAN, FIDENBERG, A. M. C. M., EIK, H. G. VAN, and LEIJNEN, R. The incorporation of ^{55}Fe , ^3H leucine and ^{32}P inorganic phosphate in ferritin during the perfusion of isolated rat livers. *Biochim. biophys. Acta* 273 243-247 (1972).
- 26 LANE, R. S. Iron uptake by rabbit reticulocytes. *Br. J. Haemat.* 24 343-353 (1973).
- 27 LANE, R. S. and FRICH, C. A. The *in vivo* plasma clearance of iron from transferrin of low and high iron saturation. *Clin. Sci.* 58, 783-793 (1970).
- 28 LARSSON, A. Ribonucleotide reductase from regenerating rat liver. *Eur. J. Biochem.* 11 113-121 (1969).
- 29 MESSINGER, T. O. A comparison of the interaction of Chinese hamster fibroblasts with human and bovine transferrin, and conalbumin (chick-egg transferrin). *Biochim. biophys. Acta* 320 663-670 (1973).
- 30 MINTEN-KAAS, A. C., BERO, T., SEGLER, P. O., and SELIGER, R. Mass isolation and culture of rat Kupffer cells. *J. exp. Med.* 141 1-10 (1975).
- 31 OKADA, S., CHIFFMAN, B., and BROWN, E. B. *In vivo* evidence for the functional heterogeneity of transferrin-bound iron. IV Selective uptake by erythroid precursors of radioiron from portal vein plasma transferrin during intestinal iron absorption. *J. Lab. clin. Med.* 89 51-64 (1977).
- 32 PHILLIPS, J. L. and AZARI, P. Effect of iron transferrin on nucleic acid synthesis in phytohemagglutinin-stimulated human lymphocytes. *Cell. Immunol.* 15 94-99 (1975).
- 33 PIRELOTTO, J. V. and ZAPOLSKI, E. J. Functional heterogeneity and pH-dependent dissociation properties of human transferrin. *Biochim. biophys. Acta* 438, 766-771 (1976).
- 34 ROSSIES, E., FANT, J. and NORTON, W. Intracellular iron-binding macromolecules in HeLa cells. *Proc. natn. Acad. Sci. USA* 69 3708-3712 (1972).
- 35 SCHOLZ, R. Untersuchungen zur Radonkompartimentierung bei der hämoglobinfrei perfundierten Rattenleber. In S. ANS and SCHOLZ Stoffwechsel der isoliert perfundierten Leber 3. Konf. Ges. Biologische Chemie, Oestrich/Rheingau 1967 (Springer Berlin 1968).
- 36 TABBUTT, R. O. A study of erythropoiesis in the rat. *Exp. Cell Res.* 43, 473-483 (1967).
- 37 VERHOEF, N. J. and EIK, H. G. VAN. Isolation, characterization and function of cord-blood transferrin. *Clin. Sci. Mol. Med.* 43, 335-340 (1975).
- 38 VERHOEF, N. J., KESTER, H. C. M., NOORDHOOF, P. J. and LEIJNEN, B. *In vitro* studies on the interaction between transferrin and rat bone marrow cells (in press).
- 39 VERHOEF, N. J., KREMER, J. H. W. and LEIJNEN, B. The effect of heterologous transferrin on the uptake of iron and haem synthesis by bone marrow cells. *Biochim. biophys. Acta* 304 114-122 (1973).

- 40 VERHOEF N J and NOORDELOOS, P J Binding of transferrin and uptake of iron by rat erythroid cells *in vitro* Clin. Sci mol. Med. 52 87-96 (1977).
- 41 VERHOEF N J NOORDELOOS, P J and LEUNSE, B Heme synthetase activity in normal human and rat erythroid cells and in sideroblastic anemia. Clinica chim. Acta 87 45-53 (1978).
- 42 WESTERVELD A. VISSER, R. P. L. S. MEIRA KJIAN P and BOOTMA, D Loss of human genetic markers in man-Chinese hamster somatic hybrids. Nature new Biol. 234 20-24 (1971)
- 43 ZIMELMAN A. P., ZIMMERMAN, H J McLEAN, R., and WEINTRAUB, L. R.. Effect of iron saturation of transferrin on hepatic iron uptake. An *in vitro* study Gastroenterology 72 129-131 (1977)
- 44 ZORRALA MALLON, H. SUTTON R. N P and EMOND R. T D EB virus-specific IgM and IgG antibodies in first-degree relatives of children with acute lymphoblastic leukaemia. Archs Dis. Childh. 50 137-141 (1975)

Paraproteinemc Variety of Pure Red Cell Aplasia

Immunological Studies in 1 Patient

L. RENZOTTI, C. DOLCI, ■ PALESTRO and C. PESCHLE

Ospedale Maggiore SS. Annunziata, Savigliano
Institute of Pathology University of Turin, Turin and
Institute of Medical Pathology and ICHO IIrd Faculty
University of Naples, Naples

Key Words. PRCA PRCA and paraproteinemia IgG inhibitor to erythropoiesis
Serum M component Lymphocyte markers

Abstract. A case of adult pure red cell aplasia (PRCA) with a serum IgG inhibitor to erythropoiesis and an IgG λ M component is presented. The study of lymphocyte populations revealed a slight but definite decrease of B and EA rosettes, with dissociation between E rosettes and PHA blastic transformation of blood lymphocytes and increase of membrane IgM-bearing lymphocytes. The relationship between PRCA and paraproteinemia is discussed: it is suggested that the serum M component may derive from an immunological imbalance between T and B lymphocytes. Since survey of the literature reveals 5 similar cases, it is suggested that paraproteinemia may be the hallmark of particular variety of chronic PRCA 'type 1'.

Pure red cell aplasia (PRCA) in the adult is a relatively rare condition, the hallmark of which is represented by almost complete absence of erythroblasts in an otherwise normal marrow. Evidence has been provided by KRANTZ [3], PESCHLE *et al.* [12-14] and MARMONT *et al.* [8] indicating that adult PRCA is often an autoimmune disorder characterized by the presence of an IgG inhibitor to erythropoiesis and the possible response to immunodepressive treatment. On both physiopathological and clinical grounds, PESCHLE *et al.* [13] have recently recognized four different types

This work was supported in part by grants from Estratom, Bruxelles (No. 159-76-7B-3100), Volkswagen Foundation, Hannover and CNR, Rome (No. 75.01009 ■ No. 76.01467.01).

Received: October 4, 1977 accepted: January 6, 1978.

of PRCA Type I presence of both a serum IgG inhibitor to erythropoiesis and elevated serum erythropoietin (Ep) levels. Type II presence of a serum IgG inhibitor neutralizing Ep absence of detectable Ep activity. Type III absence of the IgG inhibitor elevated serum Ep titer 5-10% blast cells in the marrow progression to acute myeloid leukemia. Type IV possible absence of a serum IgG inhibitor in PRCA with CLL or lymphoma.

It is well established that PRCA type I or II is often associated with various immunological disorders and/or thymoma [3-13]. In this last regard it is maintained that thymic tumors may play a significant role in the autoimmune genesis of the disease.

Recently 5 PRCA cases have been reported characterized by a monoclonal gammopathy [1, 4, 6, 17, 20]. In only 1 of these cases has the presence of a serum IgG inhibitor been investigated [4]; furthermore, the different lymphocyte populations were never scrutinized.

The present report presents a case of this variety of PRCA both the IgG inhibitor, the Ep activity and the lymphocyte markers were evaluated.

Case Report

M. Marianna, aged 72, from Cambiasco (Novara), was admitted into the Savigliano Hospital in October 1975, because of severe anemia. This anemia had first been diagnosed in February 1974. Since treatment with liver extracts, vitamin B₁₂, and iron proved unsuccessful she was maintained on blood transfusions (twice/month over the last 14 months).

On admission, Hb was 7.4 g/dl, RBC 2.05×10^6 /l, WBC 11.7×10^9 /l with a normal differential count. Reticulocytes were absent in peripheral blood. Other routine blood tests were normal serum iron was 221 µg/dl, Coombs test negative, serum proteins 8.5 g/dl. Electrophoresis revealed a peak in 2 position that was shown to be due to an IgG λ B1 component by immunoelectrophoresis. Radial immunodiffusion, IgG 40 g/l, IgA 5.5 g/l, IgM 1.19 g/l.

Bone marrow samples secured from both sternum and iliac crest were hypercellular with normal granuloblasts and megakaryocytes, a few nests of plasma cells, but without erythroblasts. Liver needle biopsy was normal.

⁵⁵Fe plasma clearance, T_{1/2} = 240 min. Plasma iron turnover = 1.3 mg/day. The incorporation of ⁵⁵Fe into the hemin of circulating RBC was markedly decreased 2.6%, day 1, 4.4%, day 2, 11.6%, day 5, 13.7%, day 8, 12.4%, day 11 and 14.

Skeleton Rx series were negative. Rx examination of anterior mediastinum did not reveal any enlargement of the thymus. Oral cholecystogram, barium coema and upper gastrointestinal Rx series were also normal.

The patient was maintained on blood transfusions until all diagnostic procedures

were completed. She was then started on cyclophosphamide (300 mg/day i. v. up to total dosage of 7.2 g). This therapy did not induce a significant increase of either reticulocytes in peripheral blood or erythroblasts in marrow. Finally it was discontinued because of the onset of leukopenia.

After 20-day interval, we started a treatment with antilymphocyte globulin (ALG Uman GAL, Biagini) - 600 mg/day by intravenous drop infusion. As result, lymphocytes disappeared almost completely from blood smears, whereas a few monocytoid cells appeared: these elements were considered as atypical myelocytes, in that they were both naphthyl AS acetate esterase negative and peroxidase and Sudan black positive. Furthermore, the number of erythroblasts did not increase in marrow aspirates. The side effects of ALG were represented by fever edemas and pemphigoid lesions on the skin, which did not recede upon prednisone therapy.

The patient's condition deteriorated rapidly. She died 2 days after the end of ALG treatment.

Physiopathological Investigations

Methods

The IgG fraction from PRCA or normal serum was purified by means of the DEAE-cellulose column chromatography [14].

The serum inhibitor to erythropoiesis was assayed by means of liquid phase cultures of normal human marrow either in the presence or not of Ep and serum IgG [4, 13]: the erythropoietic activity was evaluated on the basis of the 16-hour ^{51}Fe -heme incorporation. The amount of IgG/plate was equivalent to that contained in 0.1 ml of serum, the dosage of Ep Sep III (Consangit Med. Res. Lab., Toronto) was 0.5 IU/plate.

Blood and bone marrow lymphocytes were separated on Ficoll-Hypaque gradient. Immunofluorescence staining of the surface and cytoplasm immunoglobulins was carried out according to the method of PERIZZI *et al.* [11]; E, EA, and EAC rosettes were evaluated according to the method of JORDAN *et al.* [5] modified by PALFRELO *et al.* [10]. PHA blastic transformation of lymphocytes cultured *in vitro* was studied following the procedure described by VOLANTE and NAVONE [19].

Results

Assay of Serum Inhibitor to Erythropoiesis

Control human marrow cultures: $2,504 \pm 198/2.5 \times 10^6$ nucleated cells (cpm, mean \pm SEM). Human marrow cultures + Ep + normal serum IgG $5,624 \pm 436/2.5 \times 10^6$. Human marrow cultures + Ep + PRCA serum IgG $2,794 \pm 322/2.5 \times 10^6$.

Serum Ep Levels

Ex-hypoxic polycythemic mouse assay [15-16] > 1 IU/ml of serum.

Lymphocyte Markers

Peripheral blood PHA blastic transformation 80% (n.v. 65 ± 5) E rosettes 45% (n.v. 63 ± 8) EA rosettes 14% (n.v. 25 ± 4) membrane immunofluorescence IgG 14% (n.v. 12 ± 2) IgA 34% (n.v. 3 ± 3), IgM 22% (n.v. 10 ± 3) cytoplasm immunofluorescence IgG 8% (n.v. 4 ± 2) IgA 2% (n.v. 2 ± 1) IgM 2% (n.v. 6 ± 3)

Bone marrow E rosettes 25% (n.v. 14 ± 6) EA rosettes 8% (n.v. 10 ± 4) EAC rosettes 8% (n.v. 12 ± 6) membrane immunofluorescence IgG 16% (n.v. 0.5 ± 0.5) IgA 12% (n.v. 1 ± 1) IgM 30% (n.v. 1 ± 1) cytoplasm immunofluorescence IgG 6% (n.v. 0.5 ± 0.5) IgA 4% (n.v. 1 ± 1) IgM 0% (n.v. 1 ± 1)

Discussion

The diagnosis of chronic PRCA type I was based both on the clinical and hematological data and more particularly on the presence in serum of an IgG inhibitor to erythropoiesis and elevated serum Ep activity. The outstanding feature was represented however by the serum M component. The association of PRCA with a serum paraprotein has been previously observed in 5 patients by RESEGOTTI and RICCI [17] VOYCE [20] GILBERT *et al* [1] KRANTZ and KAO [4] and LAMENAGER *et al* [6].

Relatively little attention has been devoted so far to this association, whereas that of PRCA with thymoma has been extensively investigated (confer HIRST and ROBERTSON [2] and PESCHILE *et al* [13]). The presence in PRCA of a paraproteinemia is not coincidental but represents a relevant aspect of the generally recognized association between PRCA (type I or II) and various immunological abnormalities [13]. 5 of 6 patients reported so far showed an IgG paraprotein. In all of the 5 cases investigated adequately the light chain was of lambda type, which represents the least frequent one of normal globulins.

The physiopathological significance of the paraprotein in PRCA is not yet elucidated. It seems unlikely that the paraprotein contains the inhibitor to erythropoiesis, in that KRANTZ and KAO [4] observed after cyclophosphamide therapy the disappearance of the IgG inhibitor but not of the paraprotein. Moreover M components have not been observed in the majority of cases of PRCA showing a serum IgG inhibitor to erythropoiesis [13].

The present studies on lymphocyte populations demonstrate an immu

nological unbalance between B and T lymphocytes: this abnormality may represent the mechanism underlying the onset of both PRCA and paraproteinemia. In line with this concept, MARMONT and GORI [9] suggested that thymomas may produce an immunological unbalance between T and B lymphocytes, which in turn would favor the development of agammaglobulinemia and/or PRCA. In this regard it is of interest that PRCA, agammaglobulinemia, thymoma and paraproteinemia are often associated [1 6, 7] thus again suggesting that all of these disorders may be mediated by an unbalance between B and T lymphocytes.

References

- 1 GILBERT E. F. HARLEY J. B., AKIDO, V. MINOOLI, H. F. and FUCHER, J. T. Thymoma, plasma cell myeloma, red cell aplasia and malabsorption syndrome. *Am. J. Med.* 44 820-829 (1968).
- 2 HIRST E. and ROBERTSON, T. L. The syndrome of thymoma and erythroblastic anemia. A review of 56 cases including 3 case reports. *Medicine, Baltimore* 46 225-264 (1967).
- 3 KRANTZ, S. B.: Pure red cell aplasia. *Br. J. Haemat.* 25 1-6 (1973).
- 4 KRANTZ, S. B. and KAO, V. Studies on red cell aplasia. II. Report of a second patient with an antibody to erythroblast nuclei and remission after immunosuppressive therapy. *Blood* 34 1 11 (1969).
- 5 JORDAN, M., HOLM, G. and WHIMELL, H. Surface markers on human T and B lymphocytes. II. A large population of lymphocytes forming non immune rosettes with SRBC. *J. exp. Med.* 136 207-215 (1972).
- 6 LAMCHACER, J. TAMOUY A. et BERNARD, Y. Thymome avec déficit immunitaire, érythroblastopénie, paraprotéine monoclonale et plasmocytose médullaire. *Nouv. Presse méd.* 2 1374- (1973).
- 7 LAMITE, A. T. BURROWS, B. A., and SCARFONE, S. L. Clinicopathological conference. Refractory anemia, agammaglobulinemia and mediastinal tumor. *Am. J. Path.* 27 444-452 (1957).
- 8 MARMONT A. PERCILE, C., SANGUINETI, M., and CORDORELLI, M. Pure red cell aplasia (PRCA). Response of three patients to cyclophosphamide and/or anti-lymphocyte globulin (ALG) and demonstration of two types of serum IgG inhibitors to erythropoiesis. *Blood* 45 247-261 (1975).
- 9 MARMONT A. GORI, E. Aplasia eritroblastica pura associata a timoma in un adulto. Remissione completa dopo terapia immunosoppressiva. *Minerva med.* 64 3196-3209 (1973).
- 10 PALERSTO, G., NAVONE, R., CODA, R., LEONARDO, E., POGGIO, E., and MAGGI, G. Thymic and blood lymphocytes in myasthenia gravis. An immunological study. *Neuromuscul. med.* 18 225-229 (1976).
- 11 PERINI, B., FERRARIO, M., FORNI, L., and AMANTE, L. Immunoglobulins and lymphocyte membranes. *Prog. Immunol.* 1977 95-106.

Lymphocyte Markers

Peripheral blood PHA blastic transformation 80% (n.v. 65 ± 5) E rosettes 45% (n.v. 63 ± 8) EA rosettes 14% (n.v. 25 ± 4) membrane immunofluorescence IgG 14% (n.v. 12 ± 2) IgA 3.4% (n.v. 3 ± 3) IgM 22% (n.v. 10 ± 3) cytoplasm immunofluorescence IgG 8% (n.v. 4 ± 2) IgA 2% (n.v. 2 ± 1) IgM 2% (n.v. 6 ± 3)

Bone marrow E rosettes 25% (n.v. 14 ± 6) EA rosettes 8% (n.v. 10 ± 4) EAC rosettes 8% (n.v. 12 ± 6) membrane immunofluorescence IgG 16% (n.v. 0.5 ± 0.5) IgA 12% (n.v. 1 ± 1) IgM 30% (n.v. 1 ± 1) cytoplasm immunofluorescence IgG 6% (n.v. 0.5 ± 0.5) IgA 4% (n.v. 1 ± 1) IgM 0% (n.v. 1 ± 1)

Discussion

The diagnosis of chronic PRCA type I was based both on the clinical and hematological data and more particularly on the presence in serum of an IgG inhibitor to erythropoiesis and elevated serum Ep activity. The outstanding feature was represented, however, by the serum M component. The association of PRCA with a serum paraprotein has been previously observed in 5 patients by RESEBOTTI and RIOCI [17] VOYCE [20] GILBERT *et al* [1] KRANTZ and KAO [4] and LAMENAGER *et al* [6].

Relatively little attention has been devoted so far to this association, whereas that of PRCA with thymoma has been extensively investigated (confer HURST and ROBERTSON [2] and PESCHILE *et al* [13]). The presence in PRCA of a paraproteinemia is not coincidental but represents a relevant aspect of the generally recognized association between PRCA (type I or II) and various immunological abnormalities [13]. 5 of 6 patients reported so far showed an IgG paraprotein. In all of the 5 cases investigated adequately the light chain was of lambda type, which represents the least frequent one of normal globulins.

The physiopathological significance of the paraprotein in PRCA is not yet elucidated. It seems unlikely that the paraprotein contains the inhibitor to erythropoiesis, in that KRANTZ and KAO [4] observed after cyclophosphamide therapy the disappearance of the IgG inhibitor but not of the paraprotein. Moreover M components have not been observed in the majority of cases of PRCA showing a serum IgG inhibitor to erythropoiesis [13].

The present studies on lymphocyte populations demonstrate an immu

Methotrexate and Citrovorum Factor after Histoincompatible Allogeneic Bone Marrow Transplants in Dogs

ALOIS A. GRATWOLD, MALCOLM I. BULL, ROBERT G. GRAW Jr.,
LARRY NORTON, TURID KNUTSEN

Experimental Hematology Section, Pediatric Oncology Branch, Biostatistics and
Data Management Section, Clinical Oncology Program,
and Cytogenetic Oncology Section,
Medicine Branch, National Cancer Institute, Bethesda, Md.

Key Words. Methotrexate Citrovorum factor Immunosuppression
Bone marrow transplantation Graft-versus-host disease

Abstract. Methotrexate (MTX) followed by citrovorum factor (CVF) rescue was evaluated for its effectiveness in reducing graft-versus-host disease (GVHD) in lethally irradiated dogs transplanted with bone marrow from unrelated histoincompatible donors. Animals were given no immunosuppressive therapy (group A) or a combined regimen of MTX and CVF (group AMC). These two groups were compared with a group of animals transplanted earlier given MTX alone (group AM). Animals in the AMC group lived significantly longer than the A group ($p < 0.05$). Engraftment rate, hematopoietic recovery and incidence of GVHD were similar in all three groups. Incidence of early deaths was significant in the AM group ($p < 0.05$). It is concluded that MTX combined with CVF increases survival and is an effective posttransplantation immunosuppressive regimen with minimal toxicity.

Introduction

Fatal graft versus-host disease (GVHD) is a frequent outcome of successful bone marrow transplantation in animals and in man [25-28]. Matching of donor and recipient at the major histocompatibility locus reduces severity and incidence of GVHD in rodents, dogs and man [15-20, 25]. Posttransplantation immunosuppression by drugs has been investigated as an additional approach [5-16, 21-25, 29]. Methotrexate (MTX)

- 12 PESCHILE, C. Pathophysiology of pure red cell aplasia in the adult in LITANEY and DONNER Present Problems in Hematology pp 233-238 (Excerpta Medica, Amsterdam 1974).
- 13 PESCHILE, C. MARMONT A. PERUGINI S. BERNASCONI, C. BRUNETTI, P., FONTANA, G. GINO, R. RESEGOTTI, L. RIZZO, S. C., and CONDORELLI, M. Pathophysiology and therapy of adult pure red cell aplasia (PRCA) A cooperative study. In SHIHCHI and TAKAKU International Conference on Aplastic Anemia, pp. 287-305 (Tokyo University Press, Tokyo 1977).
- 14 PESCHILE, C. MARMONT A. MARONE, G. GENOVESE, A. SASSO, F. and CONDORELLI, M. Pure red cell aplasia. Studies on an IgG inhibitor neutralizing E_p Brit J Haemat. 30 405-411 (1975).
- 15 PESCHILE, C. and CONDORELLI, M. Biogenesis of erythropoietin. Evidence for pro-erythropoietin in a subcellular fraction of kidney Science 109 910-91 (1975).
- 16 PESCHILE, C. MARONE, G., GENOVESE, A., RAPPAFORT I. A., and CONDORELLI, M. Increased erythropoietin production in anephric rats with hyperplasia of the reticuloendothelial system induced by colloidal carbon or zymosan. Blood 47 325-337 (1976).
- 17 RESEGOTTI, L. and RICCI, C. IgG paraproteinemia in a patient with pure red cell aplasia in METCALF CONDORELLI and PESCHILE. Leukemia and aplastic anemia (Il Pensiero Scientifico, Rome 1976).
- 18 RESEGOTTI, L. PALESTRO, G. DOLCI, C. POGGIO, E. e LEONARDO, E. Le popolazioni linfocitarie nelle malattie paraproteineiche. V Giornate Ematologiche di Napoli, Napoli 1977.
- 19 VOLANTE, G. e NAVONE, R. Ricerche sul comportamento *in vitro* dei leucociti del sangue periferico di ratto in presenza di stromoagglutina. Biol Lab. 21 29-44 (1968).
- 20 VOYCE, M. A. A case of pure red cell aplasia successfully treated with cobalt. Br J Haemat. 9 412-418 (1963).

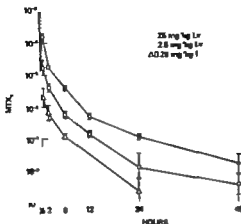


Fig 1 Serum MTX levels in normal dogs following single intravenous injections of methotrexate. Each symbol represents the median and range of 2-5 determinations in 2-5 dogs.

High-dose MTX The schedule for the posttransplantation MTX was similar to that used by other investigators in canine and human bone marrow transplantation [20, 25]. MTX at the dose of 25 mg/kg was selected after performing *in vitro* experiments which suggested that the spontaneous incorporation of tritiated thymidine into peripheral blood lymphocytes paralleled the activity of lymphocytes during the graft-versus-host reaction. This activity of DNA synthesis reflected by thymidine incorporation could be suppressed by incubating the lymphocytes for 6 h in the presence of MTX at concentrations greater than 10^{-4} M [10]. This level was achieved and maintained for longer than 6 h in dogs following 5-min infusion of intravenous MTX at 25 mg/kg (Fig. 1).

In order to assure urinary alkalization with high urinary output, 500-1,000 ml Ringer's lactate were given subcutaneously and 20 ml sodium bicarbonate 8.4% in 100 ml of normal saline, intravenously 30-60 min prior to MTX. To reduce emesis, 10 mg Compazine were given intramuscularly 3 and 6 h after the MTX, an additional 10 ml of sodium bicarbonate 8.4% were given intravenously. Serum samples were drawn at 24 h after MTX infusion and assayed for MTX levels using an enzymatic assay [4]. Animals given MTX after transplantation had circulating levels of MTX in the same range as unirradiated control animals given the same dose of MTX (Fig. 1).

Criteria for engraftment and HD The day of engraftment was defined as the first day the animal had total leukocyte count of 1×10^6 cells/liter or the second day of 0.5×10^6 cells/liter after the postirradiation nadir. In most animals, bone marrow karyotype analysis was performed to confirm engraftment with previously described techniques [26]. Animals showing progressive decline in the total leuko-

was shown to increase posttransplantation survival in mice and dogs [24 27] if given early after transplantation and on a prolonged schedule [14 18]. These studies led to the use of MTX as the posttransplantation immunosuppressive drug in human allogeneic bone marrow transplantation [25]. However, in animal studies, increased survival has been associated with increased numbers of early deaths [14 18, 19 27]. Following human transplantation, MTX toxicity has been reported [22] and in the latest series of human bone marrow transplants at the National Cancer Institute, MTX doses were often withheld or modified due to its toxicity.

Previous canine studies in our laboratories of posttransplantation MTX treatment of GVHD suggested a high incidence of early deaths and graft rejections but an increase in survival in engrafted animals [unpubl]. We have therefore developed a canine model to study if by the use of the known MTX antidote citrovorum factor (CVF) [11] toxicity to the graft and the animal could be avoided and activity against GVHD maintained. In addition, we asked whether high-dose MTX permitted by the use of CVF could extend survival in engrafted animals.

Materials and Methods

Experimental design. Two groups of animals were studied in a prospectively controlled experiment and compared with a group of animals transplanted earlier (table I). Groups A and AMC included 10 pairs of dogs. Animals of each pair were randomized to either group A or AMC. In all but 3 instances, both animals of the pair were transplanted on the same day using marrow from the same donor. All animals but 2 in each group received 6×10^6 nucleated donor bone marrow cells per kilogram recipient body weight for these paired experiments. All animals were given 900 rad total body irradiation (TBI) for pretransplantation immunosuppression. Animals in the A group were not given posttransplantation immunosuppression. AMC animals were treated with MTX and CVF as outlined in table I.

Group AM includes 26 transplanted animals prepared with 1,000 rad TBI and given posttransplantation immunosuppression with MTX alone at the dose of 0.25 mg/kg as described in table I. The number of nucleated cells given in this group varied from $4-19 \times 10^6$ cells/kg with a median of 7×10^6 cells/kg.

Animals. Closed-colony randomly bred, male and female English-American foxhounds were used as donors and beagles as recipients in all experiments. Details of the preparation and selection of the animals, radiation technique, grafting procedure and posttransplantation care have been described earlier [6]. All donor-recipient pairs were mismatched for sex and were histoincompatible at the LD locus as confirmed by mixed leukocyte culture [6]. The means of the response indices donor-recipient and recipient-donor were identical in the A and AMC groups.

Table II. Results of dogs transplanted with histoincompatible allogeneic bone marrow

Group	Early deaths prior to day 10		Engraftment		Day	Rejection		GVHD		Survival, days		Median survival
	n	%	n	%		n	%	n	%			
A	10	1/10	10	5/10	50	8 (7/10)	0/5	0	5/5	3 11 12, 12, 12 ^a 13 16 ^a 17 ^a 18 ^a 59	13	17 ^a
AMC	10	0/10	0	6/10	60	9 (6-10)	2/6	3/3	4/4	14 14 16, 16, 16, 16 ^a 21 22 27 ^a 105	18	22
AM	26	8/26	30	15/26	58	7 (6-12)	8/15	11	8/8 ^a	2, 3 3, 5, 5 6, 7 18 8, 10, 12, 12 ^a 13 17 ^a 18 19 21 23 24 24 25 26 ^a 28 65 ^a 97 200 ^a 365 ^a	18	24

n = Number of dogs.

Day of engraftment, median and range in parentheses.

1 animal with GVHD later rejected the graft.

Engrafted animals.

Engrafted animals with later rejections.

Survival

Table II summarizes the results. Animals treated with MTX and CVF showed the best survival and lived significantly longer than animals with no treatment ($p < 0.05$ one-tailed test). Survival of animals in the AM group (MTX without CVF) was between that of the A and AMC groups (fig. 2).

Engraftment, as defined above, was observed in all three groups with the same frequency and with a similar pattern of hematopoietic recovery. Since the percentage of engrafted animals is the same for all groups, a comparison of survival of the engrafted animals only can be made. Survival of the animals with stable engraftments reflects more obviously in-

cyte count after engraftment with a hypocellular *post mortem* bone marrow were classified as graft rejections. Animals with a cellular bone marrow at *post mortem* examination and having had clinical signs of GVHD [6] and/or abnormal liver function tests were considered to have GVHD.

Statistical analysis Proportions were tested by FISHER's [8] exact method for testing significance in a 2×2 contingency table. Survival curves were calculated by the method of KAPLAN and MEIER [13] and were compared using GERAN's [9] generalization of the Wilcoxon test.

Results

High Dose MTX

A single intravenous dose of 25 mg/kg of MTX is approximately the LD 50 in dogs [12]. Given as described above and followed by CVF for 3 days as outlined in table I unirradiated normal dogs show only slight lethargy. 2 control animals, irradiated and transplanted with their previously cryopreserved bone marrow died of gastrointestinal complications after high-dose MTX and CVF rescue was begun in the first week after TBI. When high dose MTX and CVF rescue was delayed for 1 week after TBI no control or test animal showed significant MTX toxicity as evidenced by bloody diarrhea, the major sign of MTX toxicity in normal dogs given high-dose MTX and insufficient CVF rescue [unpubl.]

Table I Transplantation protocol

Group	Number of dogs	Preparative regimen ¹	Bone marrow dose ²	Posttransplantation immunosuppression
A	10	900 rad, 9 rad/min	6.0 (3.0-6.0)	-
AMC	10	900 rad, 9 rad/min	6.0 (3.0-6.0)	MTX + CVF ³
AM	26	1,000 rad, 9 rad/min	7.0 (4.0-19.0)	MTX ⁴

¹ Total body irradiation midline tissue exposure dose.

² Nucleated donor bone marrow cells $\times 10^6$ /kg recipient body weight median and range.

³ MTX 0.25 mg/kg intravenously on days 1, 3, 6, then beginning on the second day of a total leukocyte count of 1×10^6 cells/liter MTX 25 mg/kg weekly until day 102. CVF 1 mg/kg, given intramuscularly 6 h after each dose of MTX (0.25 and 25 mg/kg) followed by twice-daily doses for 1 day (after the first dose of MTX) for 2 days (after the second dose of MTX on day 3), and for 3 days (after the third dose of MTX on day 6 and thereafter).

⁴ MTX 0.25 mg/kg intravenously on days 1, 3, 6, 11 and then weekly until day 102.

Discussion

GVHD is a complex syndrome which in part is the consequence of the reaction of immunocompetent cells against host antigens recognized to be foreign [28]. Numerous studies have investigated the influence of various interventions on incidence and severity of GVHD. Incidence is clearly related to histocompatibility [15, 20, 25], severity, as measured by survival, can be influenced by MTX [14, 18, 24, 27]. A major problem confronting human transplantation is that no assay is available which will predict the development of GVHD and therefore identify the patient who possibly requires the most vigorous intervention with immunosuppression. Toxicity of MTX as used for bone marrow transplantation has been of continual concern and has resulted in the discontinuation of the drug during the posttransplantation period.

MTX has been shown to suppress the humoral as well as the cellular immune response [3]. Increase in the immunosuppressive activity is dose-related and directly associated with toxicity [1, 3]. CVF reduces MTX toxicity [10, 11] and in animal models this protection is not accompanied by an equivalent reduction in the immunosuppressive effects of MTX [1, 7, 10, 17]. In mice, it was shown that a 6- to 8-hour delay between MTX and CVF was necessary to permit suppression of the immune response without sacrificing the prevention of toxicity [2]. In dogs, we could show that the combined regimen of MTX and CVF resulted in a selective suppression of lymphocytes and erythrocyte precursors with complete inhibition of antibody production [10].

This controlled study was undertaken to determine whether MTX followed by CVF rescue would provide useful posttransplantation immunosuppression with minimal toxicity in canine allogeneic bone marrow transplantation. In terms of survival, the most meaningful comparison in a controlled prospective study is to compare all animals undergoing a particular regimen. Here, the combined regimen of MTX and CVF resulted clearly in increased survival above untreated animals. If engrafted animals only are considered, survival remains better in the treatment group, but due to the small number of animals in the two groups, no statistical significance could be shown. It is however difficult to compare engrafted animals only since the distinction between engrafted and nonengrafted animals is often made by an arbitrary definition (cell counts). The survival of the nonengrafted animals in the AMC group is markedly longer than in the nonengrafted animals in the A group ($p < 0.005$ one-tailed test). No

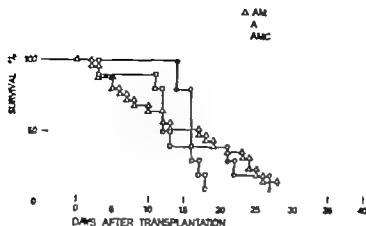


Fig 2 Survival curves for transplanted dogs.

idence of rejections and activity of GVHD Here, animals of the AMC group lived longer than animals of the A group but due to the small number of animals, no significant difference could be found. Engrafted AM animals lived slightly longer than AMC animals and significantly longer than A animals ($p < 0.05$ one-tailed test) This can be explained by the significant number of AM animals with early deaths, 8 of 26 AM animals died prior to day 10 (30%) compared to 1 animal in the A group and none in the AMC group. This high incidence of early deaths in the group receiving MTX alone is significant ($p < 0.05$) Of the engrafted animals, 2 of 6 animals rejected their graft in the AMC group and none in the A group 8 of 15 animals rejected their graft in the AM group The difference in the rejection rate between the AMC animals and either the A or AM animals is not significant.

GVHD

All animals with stable engraftments developed GVHD 1 animal in the AM group had clinical GVHD when donor karyotype cells were found in the bone marrow aspirate but then cleared all signs of GVHD at a time when karyotype analysis revealed reversion to host karyotype. This animal was sacrificed on day 200 Another animal in the AM group lived 1 year with clinically mild GVHD Autopsy showed a cellular marrow grade 1 intestinal GVHD and marked lymphoid depletion

Acknowledgements

The authors wish to thank Mrs. KATHLEEN MCCORMICK and the staff of the Hazleton Laboratories, Vienna, Va., for their excellent animal care, Dr. GERHARD R. KRUEGER for reviewing the pathology and Dr. BRUCE A. CHARNICK and Mr. JAMES C. DRAKE for their technical assistance. We are also indebted to Mr. FRANK G. STOUT and Mrs. BURBULA GRAYWOLF for their continuous help. We acknowledge the generous supply of MTX and CVP by the Drug Development Branch, National Cancer Institute, Bethesda, Md.

References

- 1 BERENBAUM, M. Prolongation of homograft survival by methotrexate with protection against toxicity by folic acid. *Lancet* **ii** 1363-1365 (1964).
- 2 BERENBAUM, M. and BROWN, L. The effect of delayed administration of folic acid on immunological inhibition by methotrexate. *Immunology* **8** 251-259 (1965).
- 3 BERENBAUM, M. Methotrexate; in GELL, COCHRAN and LACHMANN Clinical aspects of immunology (Blackwell, Oxford 1973).
- 4 BERTINO, J. and FISHER, G. Techniques for study of resistance to folic acid antagonists. *Meth. med. Res.* **10** 297-307 (1964).
- 5 BOAL, J. DACHET, R., CORCORAN, J. and WILSON, R. Modification of the graft-versus-host syndrome by antilymphocyte serum treatment of the host. *Clin. exp. Immunol.* **3** 801-808 (1968).
- 6 BULL, M., HENZEL, G. GRAY, R., KRUEGER, G. and BOWLES, C. Canine allogeneic bone marrow transplantation. *Transplantation* **22** 150-159 (1976).
- 7 D'ARCY HAN, P. REES, R., and NIVEN, J. The effect of high-dose of methotrexate associated with folic acid on the suppression of tuberculin sensitivity in guinea pigs. *Clin. exp. Immunol.* **3** 91-98 (1968).
- 8 FISHER, R. Testing of proportions. *J. R. statist. Soc. B3* 97-101 (1935).
- 9 GUPAK, E. A generalized Wilcoxon test for comparing arbitrarily singly-censored samples. *Biometrika* **52** 203-223 (1965).
- 10 GRAYWOLF, A. and DELIBEROVIC, A. Suppression of humoral immune response with methotrexate and chromomycin factor in dogs. *Current Chemotherapy Proc.* 10th Int Congr Chemother **11** 1262-1263 (1977).
- 11 GREENSPAN, E., GOLDOS, A. and SCHOTENACK, E. Studies on the mechanism of action of chemotherapeutic agents in cancer. *Cancer* **4** 619-1.5 (1951).
- 12 Hazleton Laboratories Methotrexate, acute intravenous administration in dogs, rep. No. III (Hazleton Laboratories, Vienna, Va. 1959).
- 13 KAPLAN, E. and MEIER, P. Non-parametric estimation from incomplete observations. *J. Am. statist. Ass.* **53** 457-481 (1958).
- 14 LOCYTE, H., LEVY, A., GUENTHER, D., THOMAS, E., and FERRERES, J. Prevention of delayed foreign marrow reaction in lethally irradiated mice by early administration of methotrexate. *Nature, Lond.* **196** 1110-1111 (1962).
- 15 M. TRI, G., AMIEL, J., MATSUKURA, M. and MERY, A. Restoration of hemopoietic function in the irradiated mouse by means of allogeneic bone marrow

animal given similar TBI alone without a bone marrow transplant lives as long [6]. We actually have no test and no pathognomonic histological finding to distinguish between primary and secondary rejection or early GVHD of the bone marrow in these animals living up to 16 days after transplantation with peripheral blood cell counts below 0.5×10^9 cells/liter.

6 of the 46 animals studied lived significantly longer than the other animals. They are equally distributed among the groups. If these animals were excluded from the survival analysis on the pretense that these animals for some reason were more histocompatible the results would not be different.

A direct comparison of A and AMC animals with AM animals cannot be made since the experimental conditions were not identical. However, some comparisons are possible. The incidence of early deaths in the AMF group is very high (30%) compared to about 10% of early deaths with 1 000 rad TBI and no posttransplantation immunosuppression [6]. These animals die as a result of septicemia as documented with positive blood cultures and bacterial colonization at *post mortem* examination. MTX probably contributed to these deaths by acting on peripheral cells and creating gastrointestinal lesions in addition to the lesions from the TBI. Therefore we chose a lower irradiation dose (900 rad TBI). In this study we did not see any increase in early deaths in animals given posttransplantation immunosuppression. In contrast, we achieved effective immunosuppression with increased survival above controls. It remains unclear why we had as many early deaths with doses of TBI and MTX employed without reported complications by other investigators. Additional studies in animals undergoing TBI followed by autologous bone marrow rescue would be helpful in defining the toxicity to the animals and to the infused bone marrow cells resulting from MTX with or without CVF.

This study indicates that MTX followed by CVF rescue increases survival in lethally irradiated dogs transplanted with histoincompatible bone marrow. It further confirms that MTX alone is effective but may be associated with a significant number of early deaths and may increase the incidence of eventual rejections. The addition of CVF appears to reduce early toxicity while still providing for immunosuppressive activity when compared to control animals. Thus toxicity can be reduced with full preservation of immunosuppression. Engraftment rate, rate of hematopoietic cell recovery and incidence of GVHD are not changed by either regimen. No effect on survival over 30 days was observed with high-dose MTX permitted by CVF rescue over low-dose MTX alone.

Acknowledgements

The authors wish to thank Mrs. KATHLEEN MCCORMICK and the staff of the Hareton Laboratories, Vienna, Va. for their excellent animal care, Dr. GERHARD R. KAUFER for reviewing the pathology and Dr. BRUCE A. CHARNER and Mr. JAMES C. DEAR for their technical assistance. We are also indebted to Mr. FRANK H. STOUT and Mrs. BARBARA GRATWOL for their continuous help. We acknowledge the generous supply of MTX and CVF by the Drug Development Branch, National Cancer Institute, Bethesda, Md.

References

- 1 BERENBAUM, M. Prolongation of homograft survival by methotrexate with protection against toxicity by folic acid. *Lancet* 2: 1363-1365 (1964).
- 2 BERENBAUM, M. and BROWN, I. The effect of delayed administration of folic acid on immunological inhibition by methotrexate. *Immunology* 8: 251-259 (1965).
- 3 BERENBAUM, M. Methotrexate; in GELL, COOMBS and LACHMAN *Clinical aspects of immunology* (Blackwell, Oxford 1975).
- 4 BERTHO, J. and FISCHER, G. Techniques for study of resistance to folic acid antagonists. *Meth. med. Res.* 10: 297-307 (1964).
- 5 BOAK, J. DAHER, R.; CORBOY, J. and WILSON, R. Modification of the graft-versus-host syndrome by antilymphocyte serum treatment of the host. *Clin. exp. Immunol.* 3: 801-808 (1968).
- 6 BULL, M., HERZIG, G., GRAW, R., KAUFER, G. and BOWLER, C. Canine allogeneic bone marrow transplantation. *Transplantation* 22: 150-159 (1976).
- 7 D'ARCY HART, P., REES, R., and NIVEN, J. The effect of high-dose of methotrexate associated with folic acid on the suppression of tuberculin sensitivity in guinea pigs. *Clin. exp. Immunol.* 3: 91-98 (1968).
- 8 FISHER, R. Testing of proportions. *J. R. statist. Soc.* 94: 91-101 (1935).
- 9 GEHAN, E. A generalized Wilcoxon test for comparing arbitrarily singly-censored samples. *Biometrika* 52: 203-223 (1965).
- 10 GRATWOL, A. and DEINEROTH, A. Suppression of humoral immune response with methotrexate and chloroform factor in dogs. *Current Chemotherapy Proc.* 10th Int. Congr. Chemother. 11: 1263-1265 (1978).
- 11 GREENSPAN, E., GOLDEN, A., and SCHWENBACH, E. Studies on the mechanism of action of chemotherapeutic agents in cancer. *Cancer* 4: 619-125 (1951).
- 12 Hareton Laboratories. Methotrexate, acute intravenous administration in dogs, rep. No. 10 (Hareton Laboratories, Vienna, Va. 1959).
- 13 KAPLAN, E. and MEIER, P. Non-parametric estimation from incomplete observations. *J. Am. statist. Ass.* 53: 457-481 (1958).
- 14 LOCHT, H., LEVY, A., GUENTHER, D., THOMAS, E., and FRIEDMAN, J. Prevention of delayed foreign marrow reaction in lethally irradiated mice by early administration of methotrexate. *Nature, Lond.* 196: 1110-1111 (1962).
- 15 MATTEI, H., AMEL, J., H. TSUKURA, M., and MEYER, A. Restoration of hemopoietic fraction in the irradiated mouse by means of allogeneic bone marrow

animal given similar TBI alone without a bone marrow transplant lives as long [6]. We actually have no test and no pathognomonic histological finding to distinguish between primary and secondary rejection or early GVHD of the bone marrow in these animals living up to 16 days after transplantation with peripheral blood cell counts below 0.5×10^6 cells/liter.

■ of the 46 animals studied lived significantly longer than the other animals. They are equally distributed among the groups. If these animals were excluded from the survival analysis on the pretense that these animals for some reason were more histocompatible the results would not be different.

A direct comparison of A and AMC animals with AM animals cannot be made since the experimental conditions were not identical. However, some comparisons are possible. The incidence of early deaths in the AM group is very high (30%) compared to about 10% of early deaths with 1 000 rad TBI and no posttransplantation immunosuppression [6]. These animals die as a result of septicemia as documented with positive blood cultures and bacterial colonization at *post mortem* examination. MTX probably contributed to these deaths by acting on peripheral cells and creating gastrointestinal lesions in addition to the lesions from the TBI. Therefore we chose a lower irradiation dose (900 rad TBI). In this study we did not see any increase in early deaths in animals given posttransplantation immunosuppression. In contrast, we achieved effective immunosuppression with increased survival above controls. It remains unclear why we had as many early deaths with doses of TBI and MTX employed without reported complications by other investigators. Additional studies in animals undergoing TBI followed by autologous bone marrow rescue would be helpful in defining the toxicity to the animals and to the infused bone marrow cells resulting from MTX with or without CVF.

This study indicates that MTX followed by CVF rescue increases survival in lethally irradiated dogs transplanted with histoincompatible bone marrow. It further confirms that MTX alone is effective but may be associated with a significant number of early deaths and may increase the incidence of eventual rejections. The addition of CVF appears to reduce early toxicity while still providing for immunosuppressive activity when compared to control animals. Thus, toxicity can be reduced with full preservation of immunosuppression. Engraftment rate, rate of hematopoietic cell recovery and incidence of GVHD are not changed by either regimen. No effect on survival over 30 days was observed with high-dose MTX permitted by CVF rescue over low-dose MTX alone.

Acknowledgements

The authors wish to thank Mrs. KATHLEEN MCCORMICK and the staff of the Hazleton Laboratories, Vienna, V. for their excellent animal care, Dr. GERNARD R. KRUBER for reviewing the pathology and Dr. BRUCE A. CHAMBER and Mr. JAMES C. DRAKE for their technical assistance. We are also indebted to Mr. FRANK G. STOUT and Mrs. HARRARA GRAYWOLF for their continuous help. We acknowledge the generous supply of MTX and CVF by the Drug Development Branch, National Cancer Institute, Bethesda, Md.

References

- 1 BERENBAUM, M. Prolongation of homograft survival by methotrexate with protection against toxicity by folic acid. *Lancet* ii. 1363-1365 (1964).
- 2 BERENBAUM, M. and BROWN, L. The effect of delayed administration of folic acid on immunological inhibition by methotrexate. *Immunology* 8. 251-259 (1965).
- 3 BERENBAUM, M. Methotrexate in GELL, COOMES and LACHMAN Clinical aspects of immunology (Blackwell, Oxford 1975).
- 4 BERTHO, J. and FISCHER, G.: Techniques for study of resistance to folic acid antagonists. *Meth. med. Res.* 10: 297-307 (1966).
- 5 BOAL, J. DACHEZ, R.; CORBOY, J. and WILSON, R. Modification of the graft versus-host syndrome by antilymphocyte serum treatment of the host. *Clin. exp. Immunol.* 3. 801-808 (1968).
- 6 BULL, M., HERZIG, G., GRAW, R., KRUBER, G., and BOWLEY, C. Canine allogeneic bone marrow transplantation. *Transplantation* 22. 150-159 (1976).
- 7 D'ARCY HART, P., REES, R., and NIVEN, J. The effect of high-doses of methotrexate associated with folic acid on the suppression of tuberculin sensitivity in guinea pigs. *Clin. exp. Immunol.* 3. 91-98 (1968).
- 8 FISHER, R. Testing of proportions. *J. R. statist. Soc.* 98. 91-101 (1935).
- 9 GRIHAM, E. A generalized Wilcoxon test for comparing arbitrarily singly-censored samples. *Biometrika* 52. 203-223 (1965).
- 10 GU THOM, A. and DEHNEROTH, A. Suppression of humoral immune response with methotrexate and citrovorum factor in dogs. *Current Chemotherapy Proc.* 10th Int. Congr. Chemother. 11. 1263-1263 (1978).
- 11 GREENFAX, E.; GOLDB, A., and SCHOENBACH, E. Studies on the mechanism of action of chemotherapeutic agents in cancer. *Cancer* 4. 619-125 (1951).
- 12 Hazleton Laboratories. Methotrexate, acute intravenous administration in dogs, rep. No. 10 (Hazleton Laboratories, Vienna, Va. 1959).
- 13 KARLAN, E. and MILLER, F. Non-parametric estimation from incomplete observations. *J. Am. statist. Ass.* 53. 437-481 (1958).
- 14 LOCATE, H., LEVY, A., GUENTHER, E., THOMAS, E., and FERRERES, J. Prevention of delayed foreign marrow reaction in lethally irradiated mice by early administration of methotrexate. *Nature, Lond.* 196. 1110-1111 (1962).
- 15 MITHÉ, G., AMTEL, J.; IIZUKURA, M., and MRAY, A. Restoration of hemopoietic function in the irradiated mouse by means of allogeneic bone marrow

- grafts from several donors of different strains. *Br J Haemat.* 10 257-263 (1964)
- 16 OWENS, A. and SANTOS, G. The effect of cytotoxic drugs on graft versus-host disease in mice. *Transplantation* 11 378-382 (1971).
- 17 SOLODOFF M and NEWBOULD B.. Suppression of experimental allergic encephalomyelitis by methotrexate with folic acid rescue. *Arthritis Rheum.* 13 350 (1970)
- 18 STORB R, EPSTEIN R., GRAHAM, T. and THOMAS, E. Methotrexate regimens for control of graft versus-host disease in dogs with allogeneic marrow grafts. *Transplantation* 9 40-246 (1970)
- 19 STORB, R., GRAHAM, T. SIEGURA, E. and THOMAS, E.. Treatment of canine graft versus-host disease with methotrexate and cyclophosphamide following bone marrow transplantation from histoincompatible donors. *Transplantation* 10 165-169 (1970).
- 20 STORB, R. RUDOLPH, R. KOLB, H., GRAHAM, T., MICKELSON E. ERICKSON, V. LERNER, K. KOLB, H. and THOMAS, E.. Marrow grafts between DLA-matched canine littermates. *Transplantation* 15 92-100 (1973)
- 21 STORB, R. KOLB, H. GRAHAM T. WEIDEN P. and THOMAS, E. Treatment of established graft versus-host disease in dogs by antithymocyte serum or prednisone. *Blood* 42 601-609 (1973)
- 22 STORB, R. THOMAS, E. BUCKNER, C. CLIFT R. JOHNSON, F. FEITER, A. GLUCKSBERG, H. GIBBLETT E. LERNER, K., and NEUMAN P.. Allogeneic marrow grafting for treatment of aplastic anemia. *Blood* 43 157-180 (1974)
- 23 STORB, R. GLUCKSMAN, E. THOMAS, E. BUCKNER, C. CLIFT R. FEITER, A. GLUCKSBERG, H. GRAHAM, T. JOHNSON F. LERNER, K. NEUMAN P. and OCIE, H. Treatment of established human graft versus-host disease by antithymocyte globulin. *Blood* 44 57-75 (1974)
- 24 THOMAS, E., COLLINS, J. HERMAN E., and FERLBECK, J. Marrow transplants in lethally irradiated dogs given methotrexate. *Blood* 19 17-228 (1962).
- 25 THOMAS, E. STORB, R. CLIFT R. FEITER, A. JOHNSON, F. NEUMAN P. LERNER, K. GLUCKSBERG, H., and BUCKNER, C. Medical progress. bone marrow transplantation. *New Engl. J Med* 292 832-843 895-902 (1975).
- 26 TJO, J. and WANG, J.. Chromosome preparations of bone marrow cells without prior *in vitro* culture or *in vivo* colchicine administration. *Stain Technol* 37 17-20 (1962)
- 27 UFFHOLT E. Alteration of homograft reaction by A methopterin in lethally irradiated mice treated with homologous marrow. *Proc. Soc. exp. Biol. Med* 99 651-653 (1958)
- 28 VAN BEKKUM E. Radiation chimeras (Academic Press, New York 1967).
- 29 VAN BEKKUM, D. LEDNEY G. BALNER, H. VAN PUTTEN L., and DE VRIES, M. Suppression of secondary disease following foreign bone marrow grafting with antilymphocyte serum. Ciba Foundation Group 29 (Churchill, London 1967)

A New Method for the Detection of the Plasminogen Activator Content of Vein Walls

R. H. JOHNSON and AVERIL O. MANSFIELD

Royal Southern Hospital, Liverpool

Key Words. Plasminogen activator ¹²⁵I Fibrinogen

Abstract The plasminogen activator content of vein wall has been measured by estimating the radio-activity released from ¹²⁵I-tagged fibrin clot by incubating that clot with known weight of the vein wall. This method could be used in the investigation of the fibrinolytic system of the body in its normal distribution and in diseases of the vascular system.

Introduction

The presence of the fibrinolytic activity of a normal tissue was first demonstrated by PERMIN [8] in 1950. He incubated pieces of various animal tissues on a fibrin plate and demonstrated areas of lysis of the fibrin around the tissues. This method was refined by TODD [10] in 1959. He incubated frozen sections of tissues from postmortem specimens on a fibrin sheet and demonstrated the presence of fibrinolytic activity around veins, venules and pulmonary arteries which appeared to be associated with endothelium. TODD's fibrin autograph technique was further modified by PANDOLFI *et al.* [7] who showed the fibrinolytic activity to be localised to the vasovasorum of veins in the arms and legs rather than the endothelium. They also showed, by a semi-quantitative assessment, that fibrinolytic activity of vein walls was higher in arms than in legs and that the fibrinolysis enhanced by stasis was also higher in arms.

However the fibrin autograph technique even as modified by PANDOLFI *et al.* [7] gives only a rough guide to the plasminogen activator content of a vein wall. Our method releases radio-isotopes from the fibrin plate and these isotopes can be accurately measured.

- grafts from several donors of different strains. *Br J Haemat.* 10 257-263 (1964)
- 16 OWENS, A and SANTOS, G The effect of cytotoxic drugs on graft versus-host disease in mice. *Transplantation* 11 378-382 (1971)
 - 17 SOXOLOFF M and NEWBOULD B Suppression of experimental allergic encephalomyelitis by methotrexate with folic acid rescue. *Arthritis Rheum.* 13 350 (1970)
 - 18 STORB, R, EPSTEIN R., GRAHAM T and THOMAS, E. Methotrexate regimens for control of graft versus-host disease in dogs with allogeneic marrow grafts. *Transplantation* 9 40-246 (1970)
 - 19 STORB, R., GRAHAM, T, SHIMURA, R., and THOMAS, E. Treatment of canine graft versus-host disease with methotrexate and cyclophosphamide following bone marrow transplantation from histoincompatible donors. *Transplantation* 10 165-169 (1970)
 - 20 STORB, R., RUDOLPH, R., KOLB, H, GRAHAM, T, NICKELSON, E, ERICKSON V, LERNER, K., KOLB, H., and THOMAS, E. Marrow grafts between DLA matched canine littermates. *Transplantation* 15 92-100 (1973).
 - 21 STORB, R., KOLB, H, GRAHAM, T, WEIDEN P and THOMAS, E. Treatment of established graft versus-host disease in dogs by antithymocyte serum or prednisone. *Blood* 42 601-609 (1973)
 - 22 STORB, R., THOMAS, E, BUCKNER, C., CLIFT R., JOHNSON, F., FEFER, A., GLUCKSBERG, H, GIBLETT E, LERNER, K., and NEWMAN P. Allogeneic marrow grafting for treatment of aplastic anemia. *Blood* 43 157-180 (1974)
 - 23 STORB, R., GLUCKSMAN, E, THOMAS, E, BUCKNER, C, CLIFT R., FEFER, A., GLUCKSBERG, H., GRAHAM, T., JOHNSON F, LERNER, K., NEWMAN P and OCHS, H. Treatment of established human graft versus-host disease by antithymocyte globulin. *Blood* 44 57-75 (1974).
 - 24 THOMAS, E., COLLIER, J, HERMAN E., and FERRENT, J.. Marrow transplants in lethally irradiated dogs given methotrexate. *Blood* 19 17-228 (1962).
 - 25 THOMAS, E, STORB, R., CLIFT R., FEFER, A., JOHNSON, F, NEWMAN P, LERNER, K., GLUCKSBERG, H., and BUCKNER, C. Medical progress: bone marrow transplantation. *New Engl. J Med.* 292 832-843, 893-902 (1975).
 - 26 TSO, J and WANG, J Chromosome preparations of bone marrow cells without prior *in vitro* culture or *in vivo* colchicine administration. *Stain Technol.* 37 17-20 (1962).
 - 27 URHOEFT D Alteration of homograft reaction by A-methoptertine in lethally irradiated mice treated with homologous marrow *Proc. Soc. exp. Biol. Med.* 99 651-653 (1958)
 - 28 VAN BEKKUM D Radiation chimeras (Academic Press, New York 1967)
 - 29 VAN BEKKUM, D, LEDNEY G, BALNUR, H, VAN PUTTEN L., and DE VRIES, M Suppression of secondary disease following foreign bone marrow grafting with antilymphocyte serum. C ba Foundation Group 29 (Churchill, London 1967)

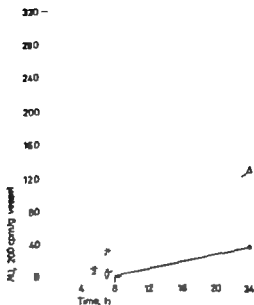


Fig 1 T show the increased separation of results between veins of high and low plasminogen activator content after 24 h incubation.

Table 1. Results from multiple tests on one vein specimen to show marked individual variation and greater correlation between the mean of few results human IVC male 25 years, transplant donor

Test No.	Weight of vein, mg	Corrected cpm	AU	Mean
1	10.5	402	10.5	106.3
	14.1	38	84.4	
3	15.3	59	19.3	
4	1.4	773	110.9	
5	14.3	381	133.2	118.5
6	15.2	110	36	
7	12.0	264	110.0	
8	12.0	475	194.7	
9	9.1	725	398.4	184.5
10	19.6	198	50.5	
11	13.5	547	202.6	
1	20.0	345	86.3	

Materials and Method

Fresh human vein samples of weight 10–20 mg each with a control sample of similar weight denatured by boiling are incubated on a fibrin clot in a commercially sterilised, disposable 5 cm Petri dish for 24 h. The fibrin clot is formed from 4 ml of a 2% solution of bovine fibrinogen (Armour) in Michaelis buffer solution [4] containing sodium azide as a bacteriostatic agent. Sufficient ^{125}I tagged fibrinogen (Radiochemical Centre, Amersham) is added so as to give 10 nCi in each 4 ml of bovine fibrinogen solution. The Petri dish is marked so as to allow identification of individual vein samples, 4 ml of the fibrinogen solution added and then clotted with an excess of thrombin (Parke Davis). The fibrinogen solution is prepared immediately before each set of tests.

The vein samples are taken from specimens removed at operation and stored in Michaelis buffer solution at 4°C until tested – usually within 4 h although storage of samples at this temperature has been shown not to affect fibrinolytic activity for a week as estimated by PANDOLFI *et al.* [6] modification of TODD's [10] technique.

After incubation for 24 h at 37°C the liquid products of lysis are aspirated using a Pasteur pipette and placed in counting tubes containing 2 ml distilled water along with the vein samples. Care is taken in aspirating the liquid products of lysis, the holes in the fibrin clot being washed with distilled water from the corresponding counting tube so that all free ^{125}I is removed. The same process is used for vein samples and the denatured controls. The radio-activity of each specimen is then recorded in a gamma counter (Nuclear Chicago model 4233) with a tube containing 2 ml distilled water only to measure background radiation. Results are given in counts per minute. After correcting these results for weight differences between the specimen and its control the radio-activity of the specimen is obtained by subtracting the figure for background radiation and the radio-activity of the control from that of the specimen.

The subtraction of radio-activity of the control vein specimen allows for non-specific actions such as the adherence of ^{125}I to the specimen.

The plasminogen activator content of a vein sample is expressed in arbitrary units (AU) where 1 AU represents the amount of plasminogen activator from 1 g of vein needed to release sufficient ^{125}I to give 200 cpm on the gamma counter. The formula used is,

$$\frac{1,000 \text{ (weight of vein specimen in mg} \times 200\text{)}}{\text{corrected cpm given by specimen}}$$

The 24-hour incubation period was selected for the following reasons. Lysis proceeds slowly at first. The longer time interval allowed the differences between vein samples with high activator and those with a low activator content to become readily apparent (fig. 1). The fibrin clot becomes unstable after 24 h and the results unreliable interval. The possibility of distortion of the results due to contamination between setting up the experiment and measuring the quantity of isotope released.

Variations in the results obtained from different samples of the same vein were found. Therefore four pieces of the same vein were always examined and the mean of the four results taken to represent the plasminogen activator content of that vein.

different individuals. The radio-activity released (by lysis) by the specimen never exceeded that released by its denatured control. In tests on the same specimens of vein using the usual fibrin clot, the radio-activity of the specimens did not exceed that of their controls.

This demonstrates that the test needs the presence of plasminogen as this was the only difference between the two sets of experiments and that it is an activator of plasminogen produced by the vein wall which is being measured.

Discussion

This method is an extension of the original method of PERMIN [8] for the demonstration of fibrinolytic activity of tissues. It enables the plasminogen activator content of a vein wall to be measured. The modification of TODD's [10] fibrin autography technique by PANDOLFI *et al* [7] gives an estimate of the degrees of activator content of a vein wall based on the amount of lysis of a fibrin sheet by a thin section of vein wall. This method is technically difficult and has a narrow range of results.

The modified Todd technique gives an estimate of the activator content of the whole thickness of the vein wall. Our method would appear to measure the activator content of that part of the specimen in contact with the fibrin. However the fibrin clot is soft enough to allow the specimen to sink into it so that activator from all parts of the specimen reach the fibrin. Early attempts by us to use thin transverse sections of vein wall, as used in the Todd technique, failed because the small amount of lysis which resulted could not be measured accurately.

As the use of ^{125}I requires a gamma-counter to calculate the results, attempts were made to measure the amounts of fibrinogen degradation products (FDP) in the area of lysis. The method used was the Wellcome FDP kit which has been shown to correlate well with the tanned red cell haemagglutination technique [1]. However this method did not prove accurate enough, especially when the amount of lysis was small and it was abandoned.

The variability of results obtained from individual pieces of the same vein (table 1) is greater than would be expected in a biological system suggesting that there are factors in the lysis of the fibrin clot which are not taken into account by the test in its present form. However by using the mean of four results, greater uniformity is consistently found. Also although we are not yet in a position to give normal values for vein wall plasminogen activator content in all parts of the body a large number of

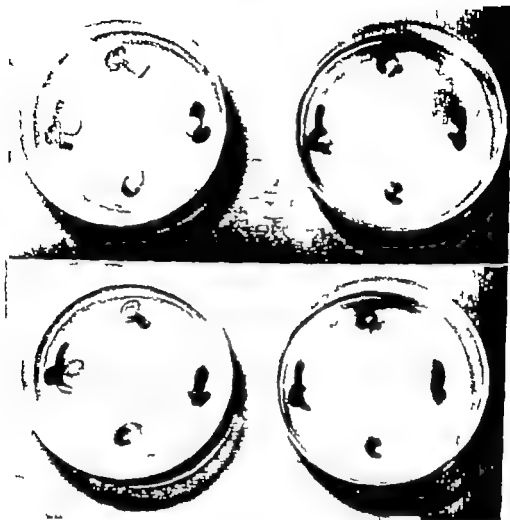


Fig 2 The fibrin plates, top with the vein specimens in place, control specimens on the right bottom after removal of the vein specimens and the product of lysis.

In multiple experiments on the same piece of vein this method was found to give more consistent results. An example of this is shown in table I

Therefore, to obtain the plasminogen activator content of a vein specimen four pieces of that vein, each with a control, are incubated on the fibrin plate described above for 24 h at 37 °C (fig.). The result of each individual specimen is calculated and the mean of the four results given as the plasminogen activator content of that vein specimen.

Effect of Using Plasminogen-Free Fibrinogen

The test was repeated 20 times with a fibrin plate made by using plasminogen free fibrinogen (Hoechst). The veins tested were from different anatomical sites and

ral distribution of vein wall plasminogen activator with reference to age, sex and site of the vein as well as in experimental grafting procedures in the arterial and venous systems.

Acknowledgements

This work was supported by grant from the Mersey Regional Health Authority. We would like to thank Prof. ROBERT SMITH and Dr ANNE HURDY SMITH, Department of Surgery University of Liverpool and Dr T. M. D. OGDEN, Consultant Physician, Liverpool Clinic, for their help and advice; M. BRADON, Photographic Department, Liverpool Area Health Authority (Teaching) for the photographs, Mr A. SANDERSON, Department of Surgery University of Liverpool, for the tables and figures and Mrs. E. A. WARD for typing the manuscript.

References

1. ERICSSON, C., STROM, T., STEWART, D. and COHEN, R. J. Evaluation of methods for the detection of quantitation of serum fibrin-fibrinogen degradation products. *Am. J. clin. Path.* 58: 394-399 (1972).
2. ISACSON, I. and NILSSON, I. M. Defective fibrinolysis in blood and vein walls in recurrent idiopathic venous thrombosis. *Acta chl. scand.* 138: 313-319 (1972).
3. KAKKAR, V. V., HOWE, C. T., NACOLAIDES, A. N., REEVEY, J. T. G. and CLARKE, M. B. Deep vein thrombosis of the leg: is there a high risk group? *Am. J. Surg.* 170: 527-530 (1970).
4. MACFARLANE, R. G. and BROWN, R. Human blood coagulation and its disorders; 3rd ed. p. 370 (Blackwell, Oxford 1963).
5. PANDOLFI, M., ISACSON, S. and NILSSON, I. M. Low fibrinolytic activity in the walls of veins: patients with thrombosis. *Acta med. scand.* 185: 1-5 (1969).
6. PANDOLFI, M., ERIKSSON, A. and NILSSON, I. M. Technical remarks on the microscopical demonstration of tissue plasminogen activator. *Thromb. Diath. haemorrh.* 27: 88-96 (1972).
7. PANDOLFI, M., ROBERTSON, B., NILSSON, I. M. and ISACSON, S. Fibrinolytic activity of human veins. *Lancet* *i*: 127-128 (1967).
8. PYRMIN, P. M. The fibrinolytic activator in animal tissue. *Acta physiol. scand.* 21: 159-167 (1950).
9. ROBERTSON, B., PANDOLFI, M., and NILSSON, I. M. Response of local fibrinolytic activity to venous occlusion of arms and legs in healthy volunteers. *Acta chir. scand.* 135: 437-440 (1977).
10. TODD, A. S. The histological localization of fibrinolytic activator. *J. Path. Bact.* 78: 281-283 (1959).

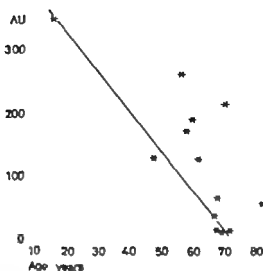


Fig 3 The relationship between age and plasminogen activator content of saphenous vein. Statistical results calculated by the regression method of least squares. $r = 0.6748$ $t = 3.1675$ $p < 0.01$

tests have been done on veins from the thigh in patients undergoing surgery for peripheral vascular disease. These results show a correlation between the age of the patient and the amount of vein wall activator content. This is statistically significant using the regression method of least squares (fig. 3). Insufficient tests have been done on veins from other sites for statistical analysis, but results in the head and neck have been found to be much higher than in the abdomen with results up to 1 200 AU for a neck vein against 700 AU for a subrenal inferior vena cava whereas results for pathological veins are consistently low varicose veins giving results of the order to 20–30 AU. These results correlate well with the clinical observations of the increased incidence of post-operative deep venous thrombosis in old age groups [3] and in the lower as opposed to the upper limbs [9]. This suggests that variations of the plasminogen activator content in different anatomical sites and different individuals may be of importance in formation of deep vein thromboses.

As deficiencies in the plasminogen activator content of vein wall have been shown in conjunction with deep venous thrombosis in the legs [2, 5] using the Pandolfi modification of Todd's technique we believe that this improved method will be of use in determining the role of vein wall plasminogen activator content in the fibrinolytic mechanisms of the body in pathological states. This method can also be used in the study of the natu

ral distribution of vein wall plasminogen activator with reference to age, sex and site of the vein as well as in experimental grafting procedures in the arterial and venous systems.

Acknowledgements

This work was supported by grant from the Mersey Regional Health Authority. We would like to thank Prof. ROBERT SMITHS and Dr ANA HARDY SMITH, Department of Surgery University of Liverpool and Mr T. M. D. GDALETTE, Consultant Physician, Liverpool Clinic, for their help and advice. Mr BRADON, Photographic Department, Liverpool Area Health Authority (Teaching) for the photographs, Mr K. SANDERSON, Department of Surgery University of Liverpool, for the tables and figures and Mrs E. A. WARD for typing the manuscript.

References

- 1 ERICKSON, C., STENO, T., STEWART, D., and CORNIN, R. J. Evaluation of methods for the detection or quantitation of serum fibrin-fibrinogen degradation products. *Am. J. clin. Path.* 53: 394-399 (1972).
- 2 ILMARINEN, J. and NILSSON, I. M. Defective fibrinolysis in blood and vein walls in recurrent idiopathic venous thrombosis. *Acta chir. scand.* 133: 313-319 (1972).
- 3 JAKKAR, V. V., HOWE, C. T., NICOLAIDES, A. N., REDFERN, J. T. G., and CLARKE, M. B. Deep vein thromboses of the leg. Is there a high risk group? *Am. J. Surg.* 120: 527-530 (1970).
- 4 MACFARLANE, R. G. and BROWN, R. Human blood coagulation and its disorders; 3rd ed. p. 370 (Blackwell, Oxford 1963).
- 5 PANDOLFI, M., ISAACSON, S., and NILSSON, I. M. Low fibrinolytic activity in the walls of veins in patients with thrombosis. *Acta med. scand.* 185: 1-5 (1969).
- 6 PANDOLFI, M., RUTENSTAD, A., and NILSSON, I. M. Technical remarks on the microscopical demonstration of tissue plasminogen activator. *Thromb. Diath. haemorrh.* 27: 88-98 (1972).
- 7 PANDOLFI, M., ROBERTSON, B., NILSSON, I. M., and ISAACSON, S. Fibrinolytic activity of human veins. *Lancet* ii: 127-128 (1967).
- 8 PERMIN, H. M. The fibrinolytic activator in animal tissue. *Acta physiol. scand.* 21: 159-167 (1950).
- 9 ROBERTSON, B. R., PANDOLFI, M., and NILSSON, I. M. Response of local fibrinolytic activity to venous occlusion of arms and legs in healthy volunteers. *Acta chir. scand.* 133: 437-440 (1972).
- 10 TODD, A. S. The histological localisation of fibrinolysin activator. *J. Path. Bact.* 73: 281-283 (1959).

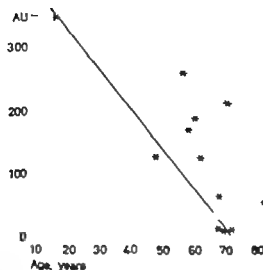


Fig 3 The relationship between age and plasminogen activator content of saphenous vein. Statistical results calculated by the regression method of least squares, $r = 0.6748$ $t = 3.1675$ $p < 0.01$

tests have been done on veins from the thigh in patients undergoing surgery for peripheral vascular disease. These results show a correlation between the age of the patient and the amount of vein wall activator content. This is statistically significant using the regression method of least squares (fig. 3). Insufficient tests have been done on veins from other sites for statistical analysis, but results in the head and neck have been found to be much higher than in the abdomen with results up to 1,200 AU for a neck vein against 700 AU for a subrenal inferior vena cava whereas results for pathological veins are consistently low varicose veins giving results of the order to 20–30 AU. These results correlate well with the clinical observations of the increased incidence of post-operative deep venous thrombosis in old age groups [3] and in the lower as opposed to the upper limbs [9]. This suggests that variations of the plasminogen activator content in different anatomical sites and different individuals may be of importance in formation of deep vein thromboses.

As deficiencies in the plasminogen activator content of vein wall have been shown in conjunction with deep venous thrombosis in the legs [2, 5] using the Pandolfi modification of Todd's technique we believe that this improved method will be of use in determining the role of vein wall plasminogen activator content in the fibrinolytic mechanisms of the body in pathological states. This method can also be used in the study of the natu-

sized to large, in part polymorphous cells with ill-defined cytoplasmic borders. The nuclei were ovoid to elliptic, in part cleaved. They showed fine chromatin structure and often had centrally placed nucleoli. Mitotic figures were frequently seen. The groups of neoplastic cells were intermingled with lymphocytes and few histiocytes and plasma cells. In silver impregnation preparations (Fig. 2) the atypical cells were grouped in small cluster-like formations with a peripheral rim of rather thick reticulin fiber bundles, or they were organized into single-cell compartments by dense net of thin silver-positive fibers.

Histopathological diagnosis so-called reticulum cell sarcoma [WHO nomenclature: reticulosarcoma cf. International histological classification of tumors, No. 14, MATH and RAFFAELLI 1975].

The patient was referred to us in February 1971. At that time he remained fully asymptomatic. Except for induration in the right supraclavicular biopsy region, the physical examination was completely normal. Clinically and radiologically there was no evidence for further tumor involvement. Radiotherapy (4,000 rad cobalt-60) to the right supraclavicular and adjacent node areas was applied to the right cervical, supraclavicular and axillary fields between May and June of 1971.

On June 15, 1971 the patient was once again referred to us with the suspicion of progressive disease. He complained of persisting anorexia of 3 months' duration with weight loss of 7 kg. He was afebrile. No peripheral adenopathy was found. The liver edge was palpated 4 cm below the costal margin, the spleen was not enlarged. There was no evidence of tumor involvement on the chest film. Films of the pelvis showed multiple small osteolytic lesions in the ileum bilaterally. A liver scan revealed multiple metastases. This was confirmed cytologically by aspiration under ultrasound control. The bone marrow was normal except for very mild increase in eosinophiles. The erythro- and erythropoiesis were normal. No tumor cells were found. The erythrocyte sedimentation rate was elevated with 46 mm in the first hour. The hemocrit was 43%. WBC were 6,300/ μ l with 8% band forms, 70% neutrophils, 2% eosinophiles, 10% monocytes and 10% lymphocytes. The BUN was 13.9 mg/dl and uric acid 4.3 mg/dl. The alkaline phosphatase was massively elevated with 230 U. The SGOT as mildly elevated with 32 U. SGPT was 16 U. The bilirubin was 0.4 mg/dl. Paper and immunoelectrophoresis were normal.

Therapy with 1.5 mg of vincristine weekly and 75 mg of prednisone daily was instituted on June 22, 1971. On July 20, maintenance therapy was instituted with vincristine 1.5 mg and cyclophosphamide 2.4 g intravenously every 4 weeks. Prednisone was given in a dose of 75 mg daily for 1 week each month. In January 1972, the cyclophosphamide therapy was changed to 300 mg daily orally for 7 days because of increasing nausea and vomiting associated with the intravenous applications. Following an episode of hematuria in November 1972, the cyclophosphamide dosage was again reduced to 150 mg daily for 7 days each month.

After initiation of chemotherapy the general condition improved rapidly. By the end of June 1971, the liver edge as palpated at the costal margin. By mid-February 1972, the alkaline phosphatase was 46 U and remained normal ever since. X-ray control of the pelvis in November 1971 showed sclerosis of the previously described osteolytic lesions. This finding also remained stable on subsequent examinations.

Clinical as well as hemoglobin, WBC and platelet controls were carried out

Non-Hodgkin's Lymphoma Terminating in Acute Myelogenous Leukemia

FRANCO CAVALLI ROLAND W SONNTAG ANDREAS ZIMMERMANN
KURT DEUBELBEISS and HANS JÜRÖ RYSEL

Divisions of Oncology and Hematology University Clinics, Inselspital and
Division of Pathology University of Berne, Berne

Key Words Lymphoma Leukemia Chemotherapy Therapy-linked neoplasia

Abstract The number of patients with non Hodgkin's lymphoma who develop acute myelogenous leukemia is relatively small. The case of a patient with histologically proven diffuse histiocytic lymphoma who died with unequivocal acute myelogenous leukemia 5 years after the lymphoma diagnosis is presented. The difficulties in differential diagnosis are cited with a review of the literature.

Patients with non Hodgkin's lymphoma (NHL) may show peripheralization (leukemic transformation) of the lymphoma during the course of the disease [18 26 27]. The development of acute myelogenous leukemia (AML) however seems to be extremely rare [3 14 17]. Since many of the terminal leukemias in lymphoma and multiple myeloma are thought to be therapy linked [6 15 16 24] information regarding the incidence of such AML during the course of NHL is of utmost importance. We describe a patient who developed AML more than 5 years after the diagnosis of diffuse histiocytic lymphoma.

Case Report

This 77 year-old white male patient first noted a swelling in the right supraclavicular region during the fall of 1970. Biopsy on November 25, 1970, revealed a diffuse histiocytic lymphoma (fig. 1). It was composed of densely packed, medium

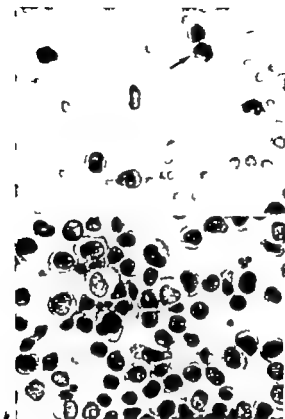


Fig 3 Peripheral blood (a) and bone marrow (b) at the time of diagnosis of AML. Arrow points to cell with an Auer rod.

monthly. On the routine control in February 1976, the general condition had deteriorated slightly and the patient complained of increasing fatigue. He was afebrile. There was no peripheral adenopathy, splenomegaly or hepatomegaly. The hemoglobin had fallen to 9.3 g/dl, platelets were 112,000/ μ l. There was leukocytosis of 25,100/ μ l with 11% neutrophils, 5% band forms, 2% eosinophils, 1% basophils, 1% monocytes, 6% lymphocytes and 74% blast forms, some of them with Auer rods (fig 3). The diagnosis of AML was made based on the presence of Auer rods and histochemical studies of peripheral blood and bone marrow smears. The great majority of the blast cells revealed positive reactions for peroxidase, Sudan black B and naphthol-ASD-chloroacetate esterase. PAS staining was only slightly and diffusely positive. In view of the advanced age of this patient and the relative lack of symptoms, no specific leukemia therapy was instituted. The patient expired 3 weeks later.

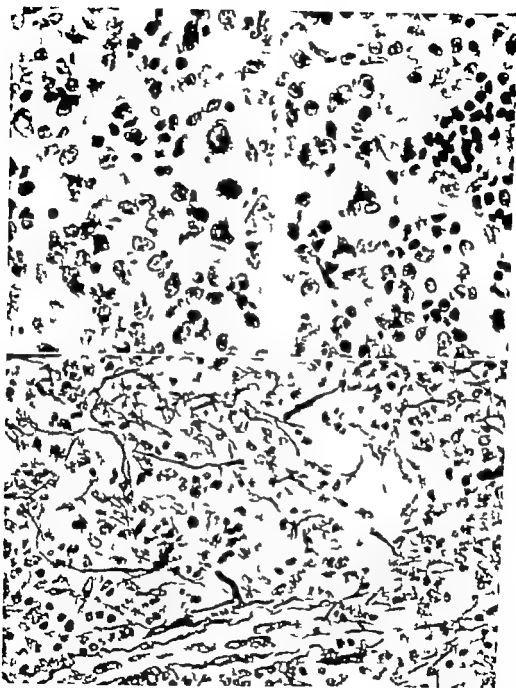


Fig 1 Highly cellular malignant neoplastic tissue with medium-sized to large, in part polymorphous cells with ill-defined cytoplasmic borders. HE. $\times 800$.

Fig 2 The atypical neoplastic cells are grouped in small cluster like formations by reticulin fibers. Silver impregnation, $\times 500$.

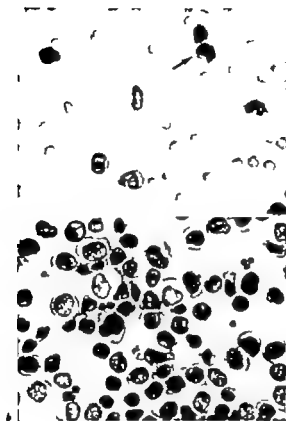


Fig 3 Peripheral blood (a) and bone marrow (b) at the time of diagnosis of AML. Arrow points to cell with an Auer rod.

monthly. On the routine control in February 1976, the general condition had deteriorated slightly and the patient complained of increasing fatigue. He was afebrile. There was no peripheral adenopathy, splenomegaly or hepatomegaly. The hemoglobin had fallen to 9.3 g/dl, platelets were 112,000/ μ l. There was leukocytosis of 25,100/ μ l with 11% neutrophils, 5% band forms, 2% eosinophiles, 1% basophiles, 1% monocytes, 6% lymphocytes and 74% blast forms, some of them with Auer rods (fig 3). The diagnosis of AML was made based on the presence of Auer rods and histochemical studies of peripheral blood and bone marrow smears. The great majority of the blast cells revealed positive reactions for peroxidase, Sudan black B and naphthol ASD-chloroacetate esterase. PAS staining was only slightly and diffusely positive. In view of the advanced age of this patient and the relative lack of symptoms, no specific leukemia therapy was instituted. The patient expired 3 weeks later.

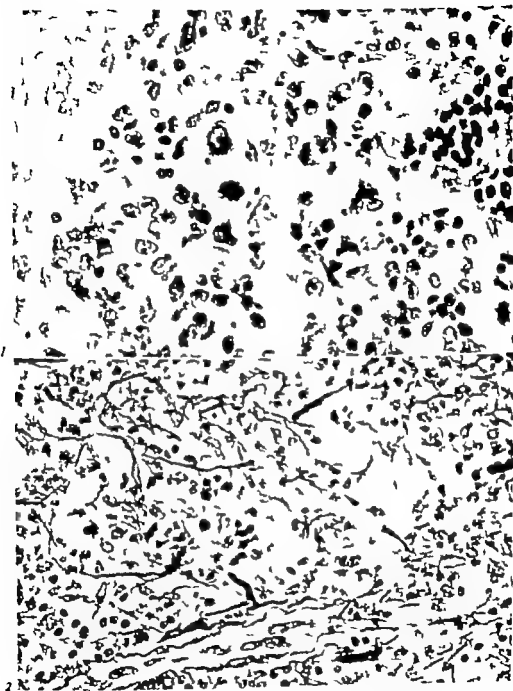


Fig 1 Highly cellular malignant neoplastic tissue with medium-sized to large in part polymorphous cells with ill-defined cytoplasmic borders. HE, $\times 800$.

Fig 2 The atypical neoplastic cells are grouped in small cluster like formations by reticulin fibers. Silver impregnation, $\times 500$.

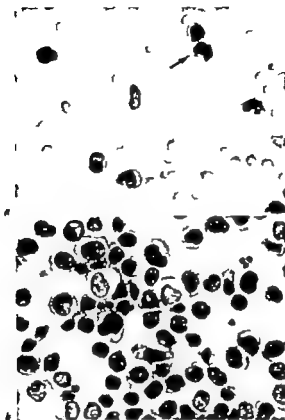


Fig 3 Peripheral blood (a) and bone marrow (b) at the time of diagnosis of AML. Arrow points to cell with an Auer rod.

monthly. On the routine control in February 1976, the general condition had deteriorated slightly and the patient complained of increasing fatigue. He was afebrile. There was no peripheral adenopathy, splenomegaly or hepatomegaly. The hemoglobin had fallen to 9.3 g/dl, platelets were $112,000/\mu\text{l}$. There was leukocytosis of $25,100/\mu\text{l}$ with 11% neutrophils, 5% band forms, 2% eosinophils, 1% basophils, 1% monocytes, 6% lymphocytes and 74% blast forms, some of them with Auer rods (Fig. 3). The diagnosis of AML was made based on the presence of Auer rods and histochemical studies of peripheral blood and bone marrow smears. The great majority of the blast cells revealed positive reactions for peroxidase, Sudan black B and naphthol-ASD-chloroacetate esterase. PAS staining was only slightly and diffusely positive. In view of the advanced age of this patient and the relative lack of symptoms, no specific leukemia therapy was instituted. The patient expired 3 weeks later.

Discussion

In contrast to Hodgkin's disease [21-24] and multiple myeloma [15-20] there are very few well-documented cases of NHL terminating in AML [3, 14, 28]. Involvement of the blood, however, is relatively common in NHL [9, 18, 21, 27]. The incidence of peripheralization late in the course of NHL [13] is not exactly known. By defining a leukemic change as a leukocyte count over 30,000 μ l with a preponderance of malignant lymphocytic cells, ROSENBERG *et al* [19] found a leukemic phase in 8.6% of patients with giant follicular lymphoma, 12.6% with lymphosarcoma and 2.4% with reticulum cell sarcoma.

The term lymphosarcoma cell leukemia is sometimes used for this phase in lymphocytic lymphoma [12]. KAPADIA and KAPLAN [14] recently reviewed 6 cases of NHL (lymphocytic type) terminating in AML. They described 1 patient with simultaneous occurrence of both diseases. In lymphocytic lymphoma it is frequently easy to distinguish peripheralization of the lymphoma from the development of AML on morphological grounds alone [3]. This distinction may be more difficult in histiocytic lymphoma, especially because other disorders with a similar clinical picture must be considered. The most difficult to differentiate from leukemic transformation are granulocytic sarcoma (chloroma) [5, 7, 11, 29] and leukemic reticuloendotheliosis [4, 10, 23]. Chloromas may be confused with histiocytic lymphomas on histological sections. Cytochemical staining with chloroacetate ASD esterase may be necessary to establish the correct diagnosis [30]. The confusion caused by nonstandardized terminology adds to this problem. In a recent review of acute leukemia in nonleukemic hematopoietic diseases, BLOOMFIELD and BRUNNING [3] stated: "Although a number of cases of histiocytic lymphoma have been reported to terminate in ANLL, none of these have been well enough studied to be sure that the initial diagnosis was correct or that peripheralization had not occurred."

In our case, the morphological features and the histochemical pattern of the leukemic cells with Auer rods are typical for AML. The term AML is used here to include acute myeloblastic, promyelocytic and myelomonocytic leukemia. The interval between the diagnosis of histiocytic lymphoma and AML (5.25 years) is much too long for a possible diagnosis of primary chloroma in the first biopsy. Therefore, this could be the first described case of true AML to develop during the course of histiocytic lymphoma.

At the present time, it is not clear why AML is less frequently seen in patients with NHL than in those with Hodgkin's disease or multiple myeloma. The mean interval between the diagnosis of Hodgkin's disease and the development of AML is 6.5 years [21]. The survival of patients with disseminated NHL is much poorer than of those with Hodgkin's disease [13]. This could be one explanation for the relative rarity of terminal leukemia in NHL. Our patient first received radiotherapy and subsequently chemotherapy. In Hodgkin's disease this sequence (radiotherapy followed by chemotherapy) has been shown to carry the highest risk for developing a second neoplasm [1]. Furthermore, our patient received moderately intensive chemotherapy for almost 5 years. Prolonged cytotoxic chemotherapy is now linked with AML in many malignant and nonmalignant disorders [1, 15, 16, 22]. Intensive chemotherapy has recently dramatically improved the prognosis for patients with advanced histiocytic lymphoma [8, 25]. The necessity of long-term maintenance chemotherapy after achieving complete remission is a problem which must be solved with consideration of possible increase in secondary neoplasia.

References

1. ARSENAULT J. C., SPONZO, R. W. *et al.* Non-lymphomatous malignant tumors complicating Hodgkin's disease: possible association with intensive therapy. *New Engl. J. Med.* 278: 1119-1122 (1977).
2. BEUTLER, E. The development of acute myelogenous leukemia in a patient with reticulum-cell lymphoma. *Ann. Intern. Med.* 40: 1217-1222 (1954).
3. BLOOMFIELD, C. D. and BRUNING, R. D. Acute leukemia as a terminal event in nonleukemic hematopoietic disorders. *Semin. Oncol.* 3: 297-317 (1976).
4. BOUWMEESTER, B. A., WESTERMAN, B. K., and DOORN, C. A. Leukemic reticuloendotheliosis. *Blood* 13: 609-630 (1958).
5. BRANNAN, D. Chloroma. *Bull. Johns Hopkins Hosp.* 38: 189-216 (1926).
6. CABELLOS, G. P., DE VITA, V. T. *et al.* Second malignancies complicating Hodgkin's disease in remission. *Lancet* i: 947-949 (1975).
7. COMINO, B. E., F. YEH, A. W. and CARTER, F. Myeloblastoma preceding blood and marrow evidence of acute leukemia. *Cancer* 18: 233-258 (1965).
8. DE VITA, V. T., CABELLOS, G. P. *et al.* Advanced diffuse histiocytic lymphoma, potentially curable disease. *Lancet* i: 248-250 (1975).
9. DECK, F., BLOOMFIELD, C. D. and BRUNING, R. D. Incidence, cytology and histopathology of non-Hodgkin's lymphoma in the bone marrow. *Cancer* 33: 1382-1398 (1974).
10. FUKUDA, T. Leukemic reticulosis. *J. reticuloendoth. Soc.* 3: 117-134 (1966).
11. GRALNICK, H. R. and DETTMAR, A. Development of myeloblastoma with massive breast and ovarian involvement during remission in acute leukemia. *Cancer* 24: 746-749 (1969).

- 12 ISAACS, R. Lymphosarcoma cell leukemia *Ann. intern. Med.* **11** 657-662 (1937).
- 13 JONES, S. E. Clinical features and course of the non Hodgkin's lymphomas. *Clin. Haematol.* **3** 131-160 (1974).
- 14 KAPADIA, B. P. and KAPLAN, S. S. Simultaneous occurrence of non-Hodgkin's lymphoma and acute myelomonocytic leukemia. *Cancer* **38** 2557-2560 (1976).
- 15 KARCHNER, R. K., MILLMAN, J. A., *et al.* Previous and simultaneous cancers in patients with leukemia. *J. chron. Dis.* **27** 5-13 (1974).
- 16 LARSEN, J. and BRINCKER, H. The incidence and characteristics of acute myeloid leukemia arising in Hodgkin's disease. *Scand. J. Haematol.* **18** 197-306 (1977).
- 17 LOWENBRAUN, S., SUTHERLAND, J. C., *et al.* Transformation of reticulum cell sarcoma to acute leukemia. *Cancer* **27** 579-585 (1971).
- 18 MATHÉ, G., POUILLARD, P. *et al.* Leukemic conversion of non-Hodgkin's malignant lymphomata. *Br. J. Cancer* **31** suppl. 2, pp. 96-102 (1975).
- 19 ROSENBERG, S. A., DIAMOND, H. B. *et al.* Lymphosarcoma. a review of 1269 cases. *Medicine* **40** 31-84 (1961).
- 20 ROSENER, F. and GRÜNWALD, H. Multiple myeloma terminating in acute leukemia. Report of 12 cases and review of the literature. *Am. J. Med.* **57** 927-939 (1974).
- 21 ROSENER, F. and GRÜNWALD, H. Hodgkin's disease and acute leukemia. Report of 8 cases and review of the literature. *Am. J. Med.* **58** 339-353 (1975).
- 22 ROBERTS, M. M. and BELL, R. Acute leukemia after immunosuppressive therapy. *Lancet* **ii** 768-770 (1976).
- 23 RUDIN, A. D. *et al.* Chronic reticulolymphocytic leukemia. *Am. J. Med.* **47** 149-162 (1969).
- 24 SARAKANYAN, G. J., AL MONHIRY, H. *et al.* Acute leukemia in Hodgkin's disease. *Cancer* **33** 1369-1375 (1974).
- 25 SCHIFF, P. S., CRABNER, B. A., *et al.* Potential for prolonged disease-free survival following combination chemotherapy of non Hodgkin's lymphoma. *Blood* **43** 181-188 (1974).
- 26 SCHNITZER, P., LOESER, L. S. *et al.* Lymphosarcoma cell leukemia. *Cancer* **26** 1062-1096 (1970).
- 27 STENTERT-KROESE, W. F., CLETON, F. J. and SOMERS, R. Leukemic progression in lymphomata. *Br. J. Cancer* **31** suppl. 2, pp. 102-107 (1975).
- 28 WEISS, R. B., BRUNDMO, R. D. and KENNEDY, J. J. Lymphosarcoma terminating in acute myelogenous leukemia. *Cancer* **30** 1275-1278 (1977).
- 29 WIERNIK, P. H. and SERPICK, A. A. Granulocytic sarcoma (chloroma). *Blood* **35** 361-369 (1970).
- 30 YAM, L. T., TAVAROLI, M. and JACOBS, P. H. Differential characterization of the 'reticulum cell' in lymphoreticular neoplasms. *Am. J. clin. Path.* **64** 171-179 (1975).
- 31 ZACHAREK, L. R. and LEDMAN, J. W. Chronic lymphocytic leukemia versus chronic lymphosarcoma cell leukemia. *Am. J. Med.* **47** 75-81 (1969).

Prognostic Factors in Multiple Myeloma

A Retrospective Study Using Conventional Statistical Methods and Computer Program

YAACOV MATZNER, JOCHANAN BENBASSAT and AARON POLLIAK

Departments of Hematology and Medicine A, Hadassah University Hospital
and Hebrew University Hadassah Medical School, Jerusalem

Key Words. Multiple myeloma Prognostic factors Prognosis Computer program Statistical methods

Abstract. The prognostic significance of age, sex, ethnic origin and various laboratory data was studied retrospectively in 69 patients with multiple myeloma using conventional statistical tests and the multiple regression computerized analysis. The conventional statistical analysis confirmed that age, anemia, uremia, hypocalcaemia, hyperglobaemia, hyperuricaemia and IgA lambda type myeloma were associated with poor prognosis. The multiple regression analysis indicated that age and blood urea nitrogen levels were the only variables which significantly affect the survival of patients with multiple myeloma. A correlation was found between blood urea nitrogen levels and other laboratory data of apparent prognostic value. The differences between our results and those of other authors are discussed and it is suggested that they may in part, be due to the fact that the interplay between the various prognostic variables was not exposed in other studies.

Introduction

There is a considerable variability in the survival of patients with multiple myeloma; however, the nature of the prognostic factors and the degree to which they influence survival have not been clearly defined in this disorder [1-5].

In the present study we attempted to determine the prognostic significance of the various clinical and laboratory manifestations of multiple myeloma. In addition to conventional statistical tests, we also employed the multiple regression analysis computer program. The results of the present study suggest that age and blood urea nitrogen (BUN) are the only

- 12 ISAACS, R. Lymphosarcoma cell leukemia. *Ann. Intern. Med.* 11 657-662 (1937).
- 13 JONES, S. E. Clinical features and course of the non-Hodgkin's lymphomas. *Clin. Haematol.* 3 131-160 (1974).
- 14 KAPADIA, S. P. and KAPLAN, S. S. Simultaneous occurrence of non-Hodgkin's lymphoma and acute myelomonocytic leukemia. *Cancer* 38. 2557-2560 (1976).
- 15 KARCHNER, R. K., MELLMAN J. A., *et al* Previous and simultaneous cancers in patients with leukemia. *J. chron. Dis.* 27 5-13 (1974).
- 16 LARSEN, J. and BRENNER, H. The incidence and characteristics of acute myeloid leukemia arising in Hodgkin's disease. *Scand. J. Haematol.* 18 197-306 (1977).
- 17 LOWENBRAUN, S. SUTHERLAND J. C., *et al* Transformation of reticulum cell sarcoma to acute leukemia. *Cancer* 27 579-585 (1971).
- 18 MATHÉ, G., POUILLARD, P. *et al* Leukemic conversion of non Hodgkin's malignant lymphomata. *Br J Cancer* 31 suppl 2, pp 96-102 (1975).
- 19 ROSENBERG, S. A. DIAMOND H. D. *et al* Lymphosarcoma a review of 1269 cases. *Medicine* 40 31-84 (1961).
- 20 ROEMER, F. and GRÖNWALD, H. Multiple myeloma terminating in acute leukemia. Report of 12 cases and review of the literature. *Am. J. Med.* 57 927-939 (1974).
- 21 ROEMER, F. and GRÖNWALD H. Hodgkin's disease and acute leukemia. Report of 8 cases and review of the literature. *Am. J. Med.* 58 339-353 (1975).
- 22 ROBERTS, M. M. and BELL, R. Acute leukemia after immunosuppressive therapy. *Lancet* ii 768-770 (1976).
- 23 RUBIN A. D. *et al* Chronic reticulolymphocytic leukemia. *Am. J. Med.* 47 149-162 (1969).
- 24 SARAKANIAN G. J. AL MONDHARY II. *et al* Acute leukemia in Hodgkin's disease. *Cancer* 33 1369-1375 (1974).
- 25 SCHEIN P. S. CHARNIER, B. A., *et al* Potential for prolonged disease-free survival following combination chemotherapy of non Hodgkin's lymphoma. *Blood* 43 181-188 (1974).
- 26 SCHNITZER, P. LOESSEL, L. S., *et al*. Lymphosarcoma cell leukemia. *Cancer* 26 1082-1096 (1970).
- 27 STENFERT KROGER, W. F. CLETON, F. J. and SOMERS, R. Leukemic progression in lymphomata. *Br J Cancer* 31 suppl 2, pp. 102-107 (1975).
- 28 WEISS, R. B. BRUNTING, R. D. and KENNEDY II J. Lymphosarcoma terminating in acute myelogenous leukemia. *Cancer* 30 1275-1278 (1972).
- 29 WIERNIK, P. H. and STIMPICK, A. A. Granulocytic sarcoma (chloroma). *Blood* 35 361-369 (1970).
- 30 YAM L. T. TAVASSOLI, M. and JACOBS, P. Differential characterization of the 'reticulum cell' in lymphoreticular neoplasms. *Am. J. clin. Path.* 61 171-179 (1975).
- 31 ZACHARSKI, L. R. and LINMAN J. W. Chronic lymphocytic leukemia versus chronic lymphosarcoma cell leukemia. *Am. J. Med.* 47 75-81 (1969).

Prognostic Factors in Multiple Myeloma

A Retrospective Study Using Conventional Statistical Methods and Computer Program

YAACOV MATZNER, JOCHANAN BENBASSAT and AARON POLLACK

Departments of Hematology and Medicine A, Hadassah University Hospital
and Hebrew University-Hadassah Medical School, Jerusalem

Key Words. Multiple myeloma Prognostic factors Prognosis Computer program Statistical methods

Abstract The prognostic significance of age, sex, ethnic origin and various laboratory data was studied retrospectively in 69 patients with multiple myeloma using conventional statistical tests and the multiple regression computerized analysis. The conventional statistical analysis confirmed that age, anemia, uremia, hypoalbuminemia, hyperglobulinemia, hyperuricemia and IgA lambda type myeloma were associated with poor prognosis. The multiple regression analysis indicated that age and blood urea nitrogen levels were the only variables which significantly affect the survival of patients with multiple myeloma. A correlation was found between blood urea nitrogen levels and other laboratory data of apparent prognostic value. The differences between our results and those of other authors are discussed and it is suggested that they may in part, be due to the fact that the interplay between the various prognostic variables was not exposed in other studies.

Introduction

There is a considerable variability in the survival of patients with multiple myeloma; however the nature of the prognostic factors and the degree to which they influence survival have not been clearly defined in this disorder [1-5].

In the present study we attempted to determine the prognostic significance of the various clinical and laboratory manifestations of multiple myeloma. In addition to conventional statistical tests, we also employed the multiple regression analysis computer program. The results of the present study suggest that age and blood urea nitrogen (BUN) are the only

variables which significantly affect the survival of our patients with multiple myeloma.

Materials and Methods

All 80 patients with multiple myeloma examined and treated in the Hadassah University Hospital during the period 1959 to 1974 and followed until the end of 1975 were included in this study. The criteria for diagnosis of myeloma were based on the protocol of the Chronic Leukemia Myeloma Task Force [6]. 58 of the patients were treated with continuous melphalan (Alkeran) or cyclophosphamide (Endoxan) while 22 patients, diagnosed before 1962, were treated with urethane only. The median survival of the group of patients treated with alkylating agents was 6.5 months while that of the group treated with urethane was 24 months. The apparent differences between the groups' survival are of no statistical significance.

The following data were reviewed: age, sex, ethnic group, laboratory data at the time of diagnosis (table I) including hemoglobin levels, leukocyte and thrombocyte counts, serum calcium, uric acid, albumin, globulin (as a rough quantitative indicator of the amount of paraprotein), BUN, type of myeloma protein in the serum and urine and radiological skeletal survey. Serum calcium levels were corrected for the serum albumin concentrations employing Payne's equation [7]:

$$\text{Corrected calcium} = \text{serum calcium (mg/dl)} - \text{serum albumin (g/dl)} + 4$$

The prognostic significance of the various data was computed in 69 patients who had died during the period of the study [8], by two different methods: (A) Evaluation of the prognostic value of the data by paired statistical tests [9] i.e. the median test and the Wilcoxon Mann-Whitney test (or the Kruskal Wallis one way analysis for more than two groups). This method evaluated the prognostic significance of each factor separately ignoring other possible prognostic factors. (B) Multiple regression analysis method, implemented by the SPSS (Statistical Package for Social Sciences) computer program. The resultant regression equations had the format

$$Y = x + AV_1 + BV_2 + CV_3 + \dots + NV_m$$

where Y represents prognosis (survival), x is a constant, A, B, C, ..., N are derived values, and $V_1, V_2, V_3, \dots, V_m$ are introduced variables (i.e. prognostic factors). This computer method tested the prognostic significance of each factor while keeping all other factors constant, enabling us to evaluate not only the correlation among the various prognostic factors, but also the coefficient and significance of each factor and the significance of the additional effect arising from the introduction of each factor or group of factors into the regression equation.

Results

Paired Statistical Tests

Age

The mean age at diagnosis among our patients (61 years) and their distribution according to age (fig. 1) were similar to those reported in oth

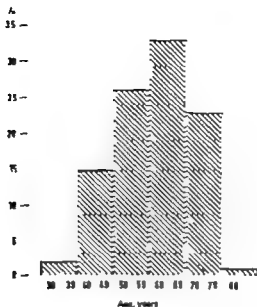


Fig 1 Age distribution (at time of diagnosis) of 80 patients with multiple myeloma.

er published series [10]. The median survival of patients below the age of 60 years was significantly longer than that of older patients (24 and 14.5 months, respectively).

Sex and Ethnic Origin

The male to female ratio among the patients was 1.2:1. European and American Jews comprised 62% of the patients while 34% were Jews of Asian and North African origin and 4% were Arabs. There were no differences in survival among patients of different sex or ethnic origin.

Immunoglobulin Class of Myeloma Protein

The relative incidence of the various types of myeloma proteins among our patients is presented in table I. There were no significant differences between the median survival of patients with IgG (23.5 months), IgA (37 months) and light chain myeloma (26 months) (fig. 2). When subgroups were compared (fig. 3) it was found that the median survival of patients with IgA/lambda paraproteinemia (16.5 months) was apparently

Table 1 Laboratory data at the time of diagnosis in 80 patients with multiple myeloma

Laboratory data		Incidence, %
Hemoglobin, g/dl	> 10.5	54
	8.5-10.5	25
	< 8.5	21
Leukocytes, per μ l	\leq 4 000	4
	\geq 4,000	96
Thrombocytes, per μ l	\leq 100,000	9
	100 000-300,000	83
	\geq 300,000	8
Corrected serum calcium mg/dl	\geq 11.5	3
	< 11.5	68
Serum uric acid, mg/dl	\geq 7.0	25
	< 7.0	75
Blood urea nitrogen, mg/dl	\leq 20	62
	21-29	19
	\geq 30	19
Type of paraprotein	IgG	56
	IgA	31
	IgD	
Type of light chain	light chain	11
	lambda	49
	kappa	51

shorter than that of patients in the remaining subgroups ($p < 0.06$). However this finding is only of borderline statistical significance because there were only 11 patients in this subgroup. There were no differences in survival between patients with or without Bence-Jones proteinuria (22 and 23 months, respectively) (fig. 4).

Blood Urea Nitrogen

The median survival of patients with BUN levels above 30 mg/dl (9 months) was significantly shorter than that of patients with lower BUN values (fig. 5). There were no significant differences between the median survival of patients with BUN levels of 21-29 mg/dl (16.5 months) and patients with normal BUN levels (24 months).

Serum Albumin and Globulin Concentrations

Patients with serum albumin concentrations less than 3 g/dl had a shorter median survival (8 months) than patients with levels above 3.5 g/dl

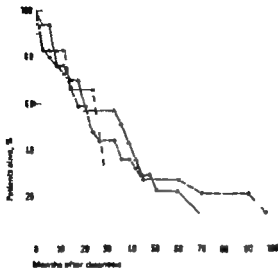


Fig 2. Survival rates according to the type of myeloma. O = IgG myeloma, Δ = IgA myeloma, ■ = light chain myeloma.

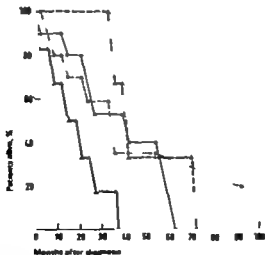


Fig 3. Survival rates according to the subtype of myeloma, showing shortened median survival of patients with IgA/lambd paraproteinaemia. O = IgG-K, ● = IgG-L, Δ = IgA-K, ▲ = IgA-L, $p < 0.05$.

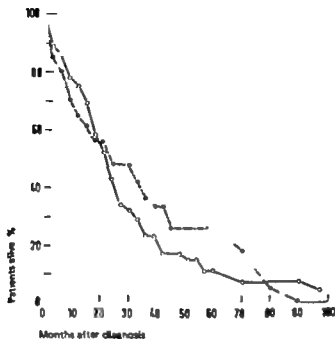


Fig 4 Survival rates of myeloma patients with (O) and without (●) Bence Jones proteinuria.

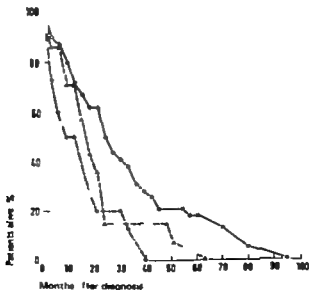


Fig 5 Survival rates according to BUN levels at time of diagnosis. □ = BUN < 20 mg/dl, ▲ = BUN 21-29 mg/dl ● = BUN ≥ 30 mg/dl $P < 0.01$

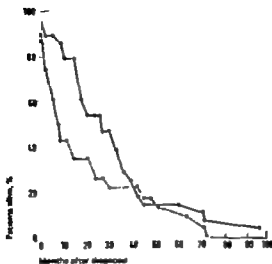


Fig 6. Survival rates according to serum albumin levels at time of diagnosis.

○ = Albumin ≥ 3.5 g/dl, ● = albumin < 3 g/dl $p < 0.05$

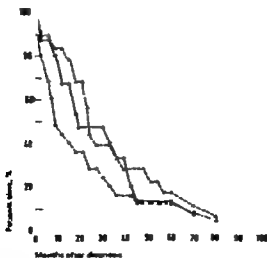


Fig 7. Survival rates according to serum globulin levels at time of diagnosis.

□ = Serum globulin ≤ 4 g/dl, △ = serum globulin 4-6 g/dl, ● = serum globulin > 6 g/dl, $p < 0.05$.

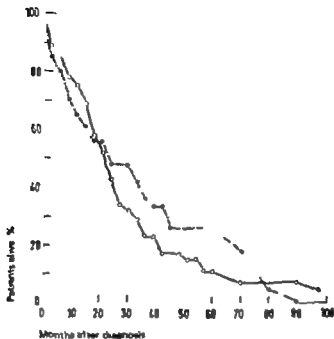


Fig 4 Survival rates of myeloma patients with (O) and without (●) Bence Jones proteinuria.

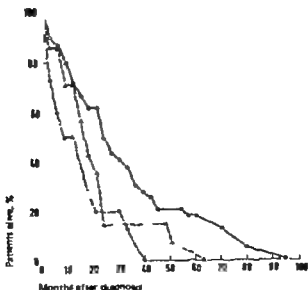


Fig 5 Survival rates according to BUN levels at time of diagnosis, □ = BUN ≤ 20 mg/dl, ▲ = BUN 21-29 mg/dl, ● = BUN ≥ 30 mg/dl $p < 0.01$

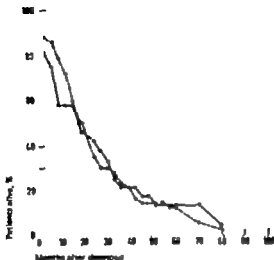


Fig 9 Survival rates according to corrected serum calcium levels at time of diagnosis. ■ = Serum calcium ≥ 11.5 mg/dl, ● = serum calcium < 11.5 mg/dl.

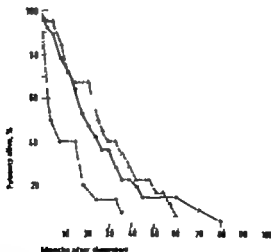


Fig 10 Survival rates according to the degree of anemia at time of diagnosis. ■ = Hemoglobin > 10.5 g/dl, ▲ = hemoglobin 8.5-10.5 g/dl, ● = hemoglobin < 8.5 g/dl, $p < 0.01$.

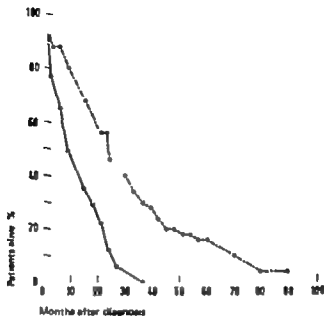


Fig 8 Survival rates according to serum uric acid levels at time of diagnosis. \circ = Uric acid ≥ 7 mg/dl, \bullet = uric acid < 7 mg/dl $p \leq 0.01$

(27 months) The median survival of patients with serum globulin levels above 6 g/dl was significantly reduced compared to that of cases with normal or moderately increased (4–6 g/dl) serum globulin levels (fig 6 7).

Serum Calcium and Uric Acid Concentrations

Serum uric acid concentrations above 7.0 mg/dl were associated with a significantly shorter median survival (fig 8). Corrected serum calcium levels had no prognostic significance (fig 9).

Hemoglobin Levels

Patients with anemia of less than 8.5 g/dl Hb had a shorter median survival (4 months) than those with moderate or no anemia. There were no significant differences in the survival of patients with normal or slightly reduced hemoglobin levels (8.5–10.5 g/dl) (fig. 10). The small number of patients with leukopenia or thrombocytopenia at diagnosis did not permit conclusions to be drawn concerning their prognostic significance.

Multiple Regression Analysis

In order to examine the effect on prognosis of all the variables (prognostic factors) and of each variable separately or in groups, all the factors

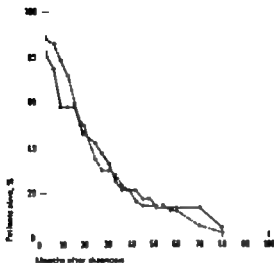


Fig 9 Survival rates according to corrected serum calcium levels at time of diagnosis. O = Serum calcium ≥ 11.5 mg/dl, ● = serum calcium < 11.5 mg/dl.

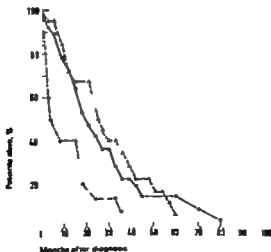


Fig 10 Survival rates according to the degree of anemia at time of diagnosis. O = Hemoglobin > 10.5 g/dl, Δ = hemoglobin 8.5-10.5 g/dl, ● = hemoglobin < 8.5 g/dl, $p < 0.01$

were entered into the equation of the multiple regression analysis and the information received is summarized below

The effect of the variables on prognosis was significant to the level of 0.002, when the multiple correlation coefficient (R^2) was 0.48. In other words, at this level of significance, all input data could account for 48% of the survival.

The only variable among the demographic data which was found to have significant effect on the prognosis was age ($p < 0.026$) with a negative coefficient of correlation ($b = -0.45$). Among the laboratory data the only variable which was found to affect the prognosis significantly was BUN ($p < 0.07$) with a negative coefficient of correlation ($b = -8.3$).

Discussion

The retrospective statistical analysis of the prognostic factors in 69 patients with confirmed multiple myeloma revealed certain similarities and some differences when compared with those reported by others [1, 3-5, 8, 11]. As in other series [1, 2, 12] sex and ethnic origin had no effect on survival while old age was associated with a poor prognosis. These findings are, however, at variance with those recorded by GALTON and PETO [3] who reported a longer median survival for female patients, without differences in prognosis among the various age groups. As in other reports [1, 3, 4, 8, 13] anemia, uremia, hypoalbuminemia, hyperglobulinemia and hyperuricemia at diagnosis were associated with a significantly shorter median survival. However, hypercalcemia, often emphasized as a poor prognostic sign [1, 8, 13] had no effect on survival in our patients. In general the type of myeloma protein did not significantly affect the median survival of the patients in this series; however, it is noteworthy that no patient with light chain myeloma survived more than 3 years. IgA/ λ myeloma was associated with the worst prognosis of all types, but the significance of this finding is questionable because of the small number of cases (11) in this group. The presence of Bence Jones proteinuria did not affect the median survival adversely. These findings differ from those recorded by ALEXANIAN *et al* [1], HANSEN *et al* [14] and the acute leukemia group II [11] who reported that IgG myeloma was associated with the best prognosis and those of GALTON and PETO [3] and HOBBS [4] who emphasized the poor prognostic significance of Bence Jones proteinuria. We have no ready explanation for these differences.

It is of interest to record that in the series of ALEXANIAN *et al* [1] in

482 patients increasing degrees of anemia, hypercalcemia, uremia and paraprotein levels were associated with a progressive shortening of life span. However as in most other studies [2, 4 5 11 12] the statistical methods used in the evaluation of the above features consisted of separate examination of each prognostic factor and the interplay between the various factors was not taken into consideration. To the best of our knowledge, the only investigations which attempted to isolate the various prognostic factors were those of GALTON and PETO [3] and KYLE and ELVEBACK [15]. GALTON and PETO [3] found that uremia, anemia and by poalbuminemia were associated with a shorter median survival but they did not use a multivariate analysis system. KYLE and ELVEBACK [15] used this technique for the following parameters: serum creatinine, calcium, albumin and globulin, hemoglobin and leukocyte levels, palpability of liver or spleen, presence of pain and age. The analysis was done on 219 cases that were divided into two groups - those who died within 2 years and those who survived 2 years or longer. The most significant variables affecting 2 years survival were serum creatinine and calcium levels.

In our study true prognostic factors were established with the aid of the multiple regression analysis computer program and the only variables which were found to have prognostic significance were uremia and old age. There also appears to be a correlation between BUN levels and other laboratory data of apparent prognostic value. Thus, when uremia develops in a patient with multiple myeloma, other abnormal laboratory findings are also likely to appear.

The apparent differences between our results and those of other authors [2, 4 5 11 12] using simple statistical methods, are probably due to their failure to expose the interrelationship between the different prognostic variables. On the other hand, our results are in keeping with those of KYLE and ELVEBACK [15] indicating the major prognostic significance of renal failure but differing in regard to the significance of age (a factor in our study) and hypercalcemia (a factor in the Mayo Clinic study but not in ours). These differences could be attributed to variations in the regression equations used in the two studies.

In the present series, as in the study by KYLE and ELVEBACK [15], we were unable to computerize data on the extent of bone lesions and the type of myeloma, or to calculate the prognosis according to tumor mass as defined by DURIE and SALMON [8] because of insufficient quantitative data. Thus, no calculations were done on the correlation of the above factors with other laboratory findings of significance.

were entered into the equation of the multiple regression analysis and the information received is summarized below.

The effect of the variables on prognosis was significant to the level of 0.002 when the multiple correlation coefficient (R^2) was 0.48. In other words, at this level of significance, all input data could account for 48% of 1st survival.

The only variable among the demographic data which was found to have a significant effect on the prognosis was age ($p < 0.026$) with a negative coefficient of correlation ($b = -0.45$). Among the laboratory data, the only variable which was found to affect the prognosis significantly was BUN ($p < 0.07$) with a negative coefficient of correlation ($b = -8.3$).

Discussion

The retrospective statistical analysis of the prognostic factors in 69 patients with confirmed multiple myeloma revealed certain similarities and some differences when compared with those reported by others [1, 3-5, 8, 11]. As in other series [1, 2, 12], sex and ethnic origin had no effect on survival while old age was associated with a poor prognosis. These findings are, however, at variance with those recorded by GALTON and PETO [3] who reported a longer median survival for female patients, without differences in prognosis among the various age groups. As in other reports [1, 3, 4, 8, 13], anemia, uremia, hypoalbuminemia, hyperglobulinemia and hyperuricemia at diagnosis were associated with a significantly shorter median survival. However, hypercalcemia, often emphasized as a poor prognostic sign [1, 8, 13], had no effect on survival in our patients. In general, the type of myeloma protein did not significantly affect the median survival of the patients in this series; however, it is noteworthy that no patient with light chain myeloma survived more than 3 years. IgA/ λ myeloma was associated with the worst prognosis of all types, but the significance of this finding is questionable because of the small number of cases (11) in this group. The presence of Bence Jones proteinuria did not affect the median survival adversely. These findings differ from those recorded by ALEXANIAN *et al* [1], HANSEN *et al* [14] and the acute leukemia group B [11] who reported that IgG myeloma was associated with the best prognosis and those of GALTON and PETO [3] and HOBBS [4] who emphasized the poor prognostic significance of Bence Jones proteinuria. We have no ready explanation for these differences.

It is of interest to record that in the series of ALEXANIAN *et al* [1] in

Idiopathic Bence-Jones Proteinuria¹

GABRIEL VIRELLA, MARIA F. LOPES-VIRELLA, J. LEVINE, MAKIO ODAWA
and JORGE GONZALEZ

Department of Basic and Clinical Immunology and Microbiology
Endocrinology Metabolism and Nutrition and Hematology Divisions
of the Department of Medicine,
Medical University of South Carolina, and the Veterans Administration Hospital,
Charleston, S. C.

Key Words. 'Idiopathic' M components Bence Jones proteinuria Diabetes

Abstract. During prospective screening for proteinuria in diabetic patients, isolated Bence-Jones proteinuria was detected in 2 cases. The first patient, 52-year-old black female, was seen for evaluation of a slow but progressive weight loss which was attributed to poor adjustment of insulin therapy. The patient gained weight after an increase of the daily insulin administration. She had plasmacytosis in bone marrow aspirate, but no other evidence of myelomatosis. The second patient, 59-year-old black male who was seen for routine evaluation of his diabetes, had no clinical or laboratory evidence of myelomatosis. Although precise definition of these cases as 'benign' or 'idiopathic' Bence-Jones proteinuria is impossible without prolonged follow-up, at the time of presentation they appeared to fit this classification. This observation is one further example that isolated Bence Jones proteinuria may be seen without any evidence of malignant B-cell dyscrasia.

The occurrence of monoclonal proteins in apparently healthy individuals has been designated by a variety of terms, the most common being 'Idiopathic' 'benign' and 'asymptomatic monoclonal gammopathies' [1, 19, 25]. WALDENSTRÖM [24] first characterized this situation with the designation of 'essential hyperglobulinemia' which soon was recognized as a relatively frequent occurrence, particularly in old age [2, 8, 20] with an

This is publication No. 197 from the Department of Basic and Clinical Immunology and Microbiology, Medical University of South Carolina. Research supported in part by ACS grant JM 161 and USPHS grant HD-09938.

Received: December 8, 1977; accepted: March 23, 1978.

Acknowledgments

We are grateful to Professor G. IZAK for advice, criticism and encouragement. The statistical evaluations were performed with the invaluable assistance of Y. FRIEDLANDER and Professor G. ZAKCK.

References

1. ALEXANIAN R, BALCERZAK, S., BONNET J D, GERAN, E. A, HAUT A, HEN LETT J S., and MONTO, R. W. Prognostic factors in multiple myeloma. *Cancer* 36: 1192-1201 (1975).
2. DAWSON, A. A and OOSTON, D. Factors influencing the prognosis in myelomatosis. *Post grad. med. J* 47: 635-638 (1971).
3. GALTOM, A. G and PETO, R. Report on the first myelomatosis trial. Analysis of presenting features of prognostic importance. *Br J Haemat.* 24: 123-139 (1973).
4. HOBBS, J. R. Monitoring myelomatosis. *Archs Intern. Med.* 135: 125-130 (1975).
5. ROMER, B. J. Multiple myeloma. A clinical review. *Med. Clin. N. Am.* 59: 375-386 (1975).
6. Committee of the Chronic Leukemia Myeloma Task Force, National Cancer Institute. Proposed guidelines for protocol studies. II. Plasma cell myeloma. *Cancer Chemother Rep* 4: 145-158 (1973).
7. PAYNE, R. B., LITTLE, A. J., WILLIAMS, R. B. and MELNER, J. R. Interpretation of serum calcium in patients with abnormal serum proteins. *Br med. J* 4: 643-646 (1973).
8. DURIE, B. G. M. and SALMON, S. E. A clinical staging system for multiple myeloma. *Cancer* 36: 842-854 (1975).
9. SEGEL, S. Nonparametric statistics for the behavioral sciences, pp. 104-127, 174-186 (McGraw-Hill, New York 1956).
10. KYLE, R. A. Multiple myeloma. Review of 869 cases. *Mayo Clin. Proc.* 50: 29-40 (1975).
11. A Cooperative Study by Acute Leukemia Group B. Correlation of abnormal immunoglobulin with clinical features of myeloma. *Archs Intern. Med.* 135: 46-52 (1975).
12. FARJANGI, M. and OSTERMAN, E. F. The treatment of multiple myeloma. *Semin. Hematol* 10: 149-161 (1973).
13. SALMON, S. E. and DURIE, B. G. M. Cellular kinetics in multiple myeloma. A new approach to staging and treatment. *Archs Intern. Med.* 135: 131-138 (1975).
14. HANSEN O. P., JENSEN B. and BIDERAEK, A. Prognosis of myelomatosis on treatment with prednisone and cytostatics. *Scand. J Haematol* 10: 282-290 (1973).
15. KYLE, R. A. and ELVERACK, L. H. Management and prognosis of multiple myeloma. *Mayo Clin Proc* 51: 751-760 (1976).

'Idiopathic' Bence-Jones Proteinuria¹

GABRIEL VIRELLA, MARIA F. LOPES-VIRELLA, J. LEVINE, MAKIO OGAWA
and JORGE GONZALEZ

Department of Basic and Clinical Immunology and Microbiology
Endocrinology Metabolism and Nutrition and Hematology Divisions
of the Department of Medicine,

Medical University of South Carolina, and the Veterans' Administration Hospital,
Charleston, S. C.

Key Words. Idiopathic' M components Bence-Jones proteinuria Diabetes

Abstract During a prospective screening for proteinuria in diabetic patients, isolated Bence Jones proteinuria was detected in 2 cases. The first patient, 52-year-old black female, was seen for evaluation of slow but progressive weight loss which was attributed to poor adjustment of insulin therapy. The patient gained weight after an increase of the daily insulin administration. She had plasmocytosis in bone marrow aspirate, but no other evidence of myelomatosis. The second patient, 59-year-old black male who was seen for routine evaluation of his diabetes, had no clinical or laboratory evidence of myelomatosis. Although precise definition of these cases as 'benign' or 'idiopathic' Bence-Jones proteinuria is impossible without prolonged follow-up, at the time of presentation they appeared to fit this classification. This observation is one further example that isolated Bence-Jones proteinuria may be seen without any evidence of malignant B-cell dyscrasia.

The occurrence of monoclonal proteins in apparently healthy individuals has been designated by a variety of terms, the most common being 'idiopathic' benign and asymptomatic monoclonal gammopathies [1, 19, 25]. WALDENSTRÖM [4] first characterized this situation with the designation of essential hyperglobulinemia which soon was recognized as a relatively frequent occurrence, particularly in old age [2, 8, 20] with an

This is publication No. 197 from the Department of Basic and Clinical Immunology and Microbiology Medical University of South Carolina. Research supported in part by ACS grant IM 161 and USPHS grant HD-09938.

Received: December 8, 1977; accepted: March 23, 1978.

Acknowledgments

We are grateful to Professor G. IZAK for advice, criticism and encouragement. The statistical evaluations were performed with the invaluable assistance of Y. FRIEDLANDER and Professor G. ZAJACK.

References

- 1 ALEXANDIAN R, BALCERZAK, S, BONNET J D, GEHAN E A, HAUT A, HEW LETT J S, and MONTO, R. W. Prognostic factors in multiple myeloma. *Cancer* 36: 1197-1201 (1975).
- 2 DAWSON A A and OOSTON D. Factors influencing the prognosis in myeloma tosis. *Post-grad med J* 47: 635-638 (1971).
- 3 GALTON A G and PETO, R.. Report on the first myelomatosis trial. Analysis of presenting features of prognostic importance. *Br J Haemat.* 24: 123-139 (1973).
- 4 HOWES, J R. Monitoring myelomatosis. *Archs Intern. Med* 135: 125-130 (1975).
- 5 ROSEN B J. Multiple myeloma. A clinical review. *Med Clin. N Am.* 59: 375-386 (1975).
- 6 Committee of the Chronic Leukemia Myeloma Task Force, National Cancer Institute. Proposed guidelines for protocol studies. II. Plasma cell myeloma. *Cancer Chemother Rep.* 4: 145-158 (1973).
- 7 PAYNE R. B., LITTLE, A. J., WILLIAMS, R. B. and MILNER, J R.. Interpretation of serum calcium in patients with abnormal serum proteins. *Br med. J* 4: 643-646 (1973).
- 8 DURIE, R. G M. and SALMON, S. E. A clinical staging system for multiple myeloma. *Cancer* 36: 842-854 (1975).
- 9 SIEGEL, S. Nonparametric statistics for the behavioral sciences, pp. 104-177, 174-186 (McGraw Hill, New York 1956).
- 10 KYLE, R. A.. Multiple myeloma. Review of 869 cases. *Mayo Clin. Proc.* 50: 79-90 (1975).
- 11 A Cooperative Study by Acute Leukemia Group B. Correlation of abnormal immunoglobulin with clinical features of myeloma. *Archs Intern. Med.* 135: 46-52 (1975).
- 12 FARHANGI, M. and OSSERMAN E. F. The treatment of multiple myeloma. *Semin. Hematol* 10: 149-161 (1973).
- 13 SALMON, S. E. and DURIE, B G M. Cellular kinetics in multiple myeloma. A new approach to staging and treatment. *Archs Intern. Med.* 135: 131-138 (1975).
- 14 HANSEN, O P, JENSEN, B. and BIDEBAEK, A. Prognosis of myelomatosis on treatment with prednisone and cytostatics. *Scand. J Haematol.* 10: 282-290 (1973).
- 15 KYLE, R. A. and ELVEBACK, L. R. Management and prognosis of multiple myeloma. *Mayo Clin. Proc.* 51: 751-760 (1976).

Idiopathic Bence-Jones Proteinuria¹

GABRIEL VIRELLA, MARIA F. LOPES-VIRELLA, J. LEVINE, MAKIO OGAWA
and JORGE GONZALEZ

Department of Basic and Clinical Immunology and Microbiology,
Endocrinology Metabolism and Nutrition and Hematology Divisions
of the Department of Medicine,

Medical University of South Carolina, and the Veterans Administration Hospital,
Charleston, S. C.

Key Words. 'Idiopathic' M components Bence Jones proteinuria Diabetes

Abstract. During prospective screening for proteinuria in diabetic patients, isolated Bence Jones proteinuria was detected in 2 cases. The first patient, 52-year old black female, was seen for evaluation of slow but progressive weight loss which was attributed to poor adjustment of insulin therapy. The patient gained weight after an increase of the daily insulin administration. She had plasmacytosis in bone marrow aspirate, but no other evidence of myelomatosis. The second patient, 59-year-old black male who was seen for routine evaluation of his diabetes, had no clinical or laboratory evidence of myelomatosis. Although precise definition of these cases as 'benign' or 'idiopathic' Bence-Jones proteinuria is impossible without prolonged follow-up, at the time of presentation they appeared to fit this classification. This observation is one further example that isolated Bence-Jones proteinuria may be seen without any evidence of malignant B-cell dyscrasia.

The occurrence of monoclonal proteins in apparently healthy individuals has been designated by a variety of terms, the most common being idiopathic 'benign' and asymptomatic monoclonal gammopathies [1, 19, 25]. WALDENSTRÖM [24] first characterized this situation with the designation of essential hyperglobulinemia which soon was recognized as a relatively frequent occurrence, particularly in old age [2, 8, 20] with an

This is publication No. 197 from the Department of Basic and Clinical Immunology and Microbiology Medical University of South Carolina. Research supported in part by ACS grant 1M 161 and USPHS grant HD-05938.

Received: December 8, 1977 accepted: March 23, 1978.

estimated incidence of 19/100 in individuals more than 95 years of age [20]. The vast majority of monoclonal proteins characterized in individuals without evidence of plasma cell dyscrasia [17] have been complete molecules of the IgG, IgA, and IgM classes. The elimination of Bence Jones proteins has been considered to be incompatible with the diagnosis of benign or idiopathic gammopathy [11]. In 1968 DAMMACCO and WALDENSTRÖM [6] reported the finding of small levels of free light chains in the urine of 41% of patients with benign monoclonal gammopathies. Their study was quantitative, however, and would not have distinguished true Bence Jones proteinuria from the elimination of normal (polyclonal) light chains known to occur in patients with plasma cell dyscrasia [22] as well as in normal individuals. Using strict methods for the characterization of Bence Jones proteins, HOBBS [11] reported negative findings in the urine of 76 patients with benign paraproteins.

The first reports of idiopathic Bence Jones proteinuria were published in 1973 [13]. However, the 2 reported cases had IgG monoclonal proteins in the serum concurrently with free λ -chains in the urine, and in that sense they were not comparable with the situation usually defined as light chain disease [21-26]. 1 year later isolated Bence Jones proteinuria was reported in a patient with antecedent colonic carcinoma and immunoglobulin deficiency, but with no other evidence of myelomatosis [5]. LINDSTRÖM and DAHLSTRÖM [14] recently studied 44 patients with benign monoclonal gammopathy, 19 of whom had Bence-Jones proteinuria in addition to a serum monoclonal component consisting of whole immunoglobulin molecules.

We report 2 additional cases of idiopathic Bence Jones proteinuria detected in a screening of urinary protein abnormalities in diabetic patients. One of them was found to have increased numbers of plasma cells in the bone marrow, whereas the other had no evidence of plasma cell malignancy.

Case Histories

Patient 1

This patient, a 52-year-old black female, presented for evaluation of weight loss. The patient had developed blurred vision and weakness in 1965. A random blood sugar was 450 mg/dL, and she was started and had remained on 45 U of NPH insulin every morning. Her initial symptoms disappeared and she felt well except for a progressive weight loss of 52 lb over the past 12 years. She has experienced no diabetic complications. Her only other problem has been mild hypertension controlled with hydrochlorothiazide. She admitted to some mild bone pain in her shins and left

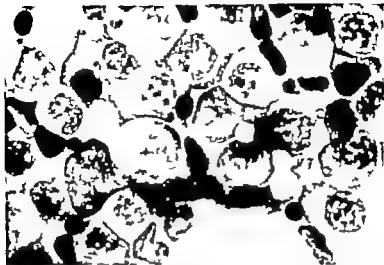


Fig 1 Bone marrow aspirate from patient L. Notice the cluster of large plasma cells with immature nuclei and occasional anucleoli. Wright stain.

rib cage only after prolonged questioning. Family history was negative for diabetes and cancer.

On physical examination, the patient was thin but appeared healthy. Height was 170 cm, weight 60 kg. Blood pressure was 154/76. Fundoscopic examination revealed bilateral macroneurysms with one exudate. Heart sounds were normal.

A random blood sugar was 510 mg/dl. The patient, weight loss was thought to be caused by inadequate insulin therapy. The haemoglobin was increased to 40 U NPH q AM and 10 U NPH q PM. A fasting blood glucose 1 week later was 112 mg/dl. The patient had gained 15 lb. in the past 4 months.

Since the patient had not been evaluated for 10 years, routine laboratory studies were performed. Serum electrolytes were as follows: sodium 141 mEq/l, potassium 3.7 mEq/l, chloride 95 mEq/l, carbon dioxide content 30 mEq/l, calcium 9.5 mg/dl, and phosphate 3.4 mg/dl. Serum creatinine was 1.3 mg/dl and creatinine clearance was 91 ml/min. 24-hour urine collections revealed proteinuria varying from 1.1 g to 69 g. Hemoglobin was 12.1 g/dl, hematocrit 37% and sedimentation rate 40 mm/h. White blood cells were 9 700 with normal differential. Cholesterol was 222 mg/dl and triglycerides 69 mg/dl. Total serum proteins were 5.8 g/dl with albumin 3.8 g/dl, α_1 -globulin 0.2 g/dl, α_2 -globulin 0.8 g/dl, β -globulin 0.9 g/dl, γ -globulin 1.3 g/dl. Immunoglobulin quantitation showed an IgG of 1,295 mg/dl, IgA of 90 mg/dl, and IgM of 96 mg/dl. No monoclonal spike was seen.

The proteinuria was initially presumed to be manifestation of diabetic renal disease. However qualitative studies (see below) revealed only $\bar{\kappa}$ -chains. Therefore,

estimated incidence of 19/100 in individuals more than 95 years of age [20]. The vast majority of monoclonal proteins characterized in individuals without evidence of plasma cell dyscrasia [17] have been complete molecules of the IgG, IgA, and IgM classes. The elimination of Bence Jones proteins has been considered to be incompatible with the diagnosis of benign or idiopathic gammopathy [11]. In 1968 DAMBIACCO and WALDENSTRÖM [6] reported the finding of small levels of free light chains in the urine of 41% of patients with benign monoclonal gammopathies. Their study was quantitative, however, and would not have distinguished true Bence Jones proteinuria from the elimination of normal (polyclonal) light chains, known to occur in patients with plasma cell dyscrasia [22] as well as in normal individuals. Using strict methods for the characterization of Bence Jones proteins, HOBBS [11] reported negative findings in the urine of 76 patients with benign paraproteins.

The first reports of idiopathic Bence-Jones proteinuria were published in 1973 [13]. However, the 2 reported cases had IgG monoclonal proteins in the serum concurrently with free A-chains in the urine, and in that sense they were not comparable with the situation usually defined as light chain disease [21-26]. 1 year later isolated Bence Jones proteinuria was reported in a patient with antecedent colonic carcinoma and immunoglobulin deficiency, but with no other evidence of myelomatosis [5]. LINDSTRÖM and DAHLSTRÖM [14] recently studied 44 patients with benign monoclonal gammopathy, 19 of whom had Bence Jones proteinuria in addition to a serum monoclonal component consisting of whole immunoglobulin molecules.

We report 2 additional cases of idiopathic Bence-Jones proteinuria detected in a screening of urinary protein abnormalities in diabetic patients. One of them was found to have increased numbers of plasma cells in the bone marrow, whereas the other had no evidence of plasma cell malignancy.

Case Histories

Patient 1

This patient, a 52-year-old black female, presented for evaluation of weight loss. The patient had developed blurred vision and weakness in 1963. A random blood sugar was 450 mg/dl, and she was started and had remained on 45 U of NPH insulin every morning. Her initial symptoms disappeared and she felt well except for a progressive weight loss of 52 lb over the past 12 years. She has experienced no diabetic complications. Her only other problem has been mild hypertension controlled with hydrochlorothiazide. She admitted to some mild bone pain in her shin and left

Patient II



Patient I

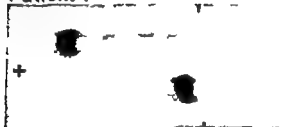


Fig 3 Cellulose acetate electrophoresis of serum and concentrated urine samples from patients I (b) and II (-). Notice the homogeneous β -fractions in the urines and the absence of homogeneous fractions in the sera.

for syphilis in 1960 and had received treatment for it in the past. The patient had undergone transurethral prostatic resection 1 year ago for benign prostatic hyperplasia. Review of systems was negative except for chronic low-back pain for the last 35 years. Family history revealed adult-onset diabetes mellitus in his mother and maternal aunt, but was negative for cancer.

On physical examination, the patient appeared healthy. Height was 183 cm and weight 72 kg. Blood pressure was 164/84. Fundoscopic examination revealed bilateral microaneurysms and a few hard exudates, but no evidence of proliferative retinopathy. The rest of the physical examination was normal.

A random blood sugar was 251 mg/dl. Serum electrolytes were as follows: sodium 133.6 mEq/l, potassium 4.7 mEq/l, chloride 91.8 mEq/l, carbon dioxide content 4.6 mEq/l, calcium 10 mg/dl, and phosphate 4.1 mg/dl. Serum creatinine was 8.9 mg/dl and creatinine clearance was 149 ml/min. Hemoglobin was 14 g/dl, hematocrit 45%, and sedimentation rate 6 mm/h. White blood cells were 8,500 with normal differential. Total serum protein was 6.50 g/dl with albumin of 3.4 g/dl, globulin 0.23 g/dl, α_1 -globulin 0.88 g/dl, β -globulin 0.79 g/dl, and γ -globulin 1.21 g/dl. Immunoglobulin quantitation showed an IgG of 990 mg/dl, IgA 140 mg/dl, and IgM 75 mg/dl. No monoclonal spike was seen. VDRL reaction was positive at 1:2

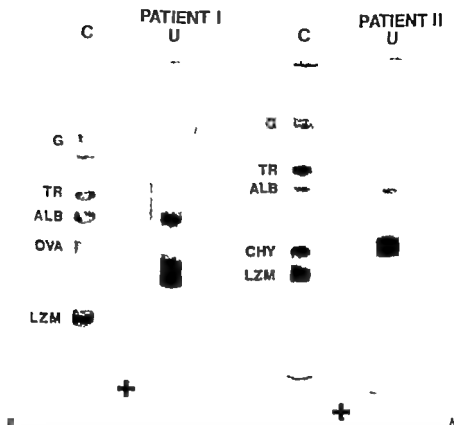


Fig 2 SDS-PAGE electrophoresis of the urinary proteins of patients I (a) and II (b). Purified proteins of known molecular weight were used as references in control (C) gels: IgG (G), transferrin (TR), serum albumin (ALB), egg albumin (OVA), chymotrypsinogen A (CHY) and human lysozyme (LZM). U = Urine

skeletal survey and bone scans were performed. Both studies were negative. A bone marrow aspiration specimen revealed plasmacytosis of 15 %. Plasma cells were seen in aggregates; they were generally large and showed immature nuclei and occasional nucleoli (fig. 1). No therapy was instituted and the patient remains asymptomatic 2 months after diagnosis. Repeat immunoglobulin quantitation showed practically identical values to those determined one year earlier.

Patient II

This patient, a 59-year-old black male with a 34-year history of diabetes mellitus, presented for evaluation of possible manifestations of his long-standing diabetes. The patient had a history of good control on a 2,200-calorie ADA diet and 20 units of NPH insulin daily. Past medical history revealed long-standing peptic ulcer disease well controlled with occasional use of antacids. He had positive serology

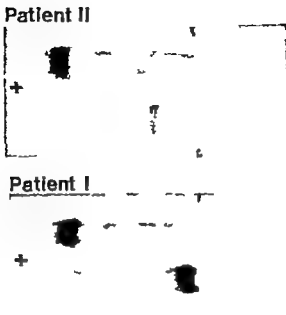
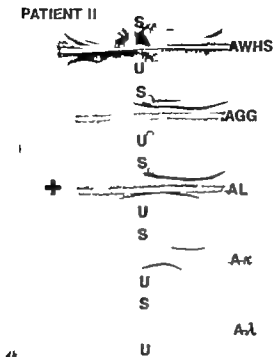


Fig 3 Cellulose acetate electrophoresis of serum and concentrated urine samples from patients I (b) and II (a). Notice the homogeneous β -fractions in the urines and the absence of homogeneous fractions in the sera.

for syphilis in 1960 and had received treatment for it in the past. The patient had undergone transurethral prostatic resection 1 year ago for benign prostatic hyperplasia. Review of systems was negative except for chronic low-back pain for the last 35 years. Family history revealed adult-onset diabetes mellitus in his mother and maternal aunt, but was negative for cancer.

On physical examination, the patient appeared healthy. Height was 183 cm and weight 77 kg. Blood pressure was 164/84. Fundoscopic examination revealed bilateral microaneurysms and a few hard exudates, but no evidence of proliferative retinopathy. The rest of the physical examination was normal.

A random blood sugar was 251 mg/dl. Serum electrolytes were as follows: sodium 133.6 mEq/l, potassium 4.7 mEq/l, chloride 91.8 mEq/l, carbon dioxide content 24.6 mEq/l, calcium 10 mg/dl, and phosphorus 4.1 mg/dl. Serum creatinine was 8.9 mg/dl and creatinine clearance was 149 ml/min. Hemoglobin was 14 g/dl, hematocrit 45%, and sedimentation rate 6 mm/h. White blood cell count 8,800 with normal differential. Total serum protein was 6.50 g/dl with albumin of 3.4 g/dl, α_1 -globulin 0.23 g/dl, α_2 -globulin 0.88 g/dl, β -globulin 0.79 g/dl, and γ -globulin 1.21 g/dl. Immunoglobulin quantitation showed an IgG of 930 mg/dl, IgA 140 mg/dl, and IgM 75 mg/dl. No monoclonal spike was seen. VDRL reaction was positive at 1:2



Special Studies

Patient 1

The abnormal proteinuria was initially detected by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE, fig. 2) [18]. Two fractions were obvious, one with the mobility of albumin, the other moving into an intermediate position between that of egg albumin (46,000 daltons) and lysozyme (15 000 daltons). This last fraction was estimated by densitometry to correspond to 57% of the total protein eliminated. Based on our previous experience with the urines of patients with monoclonal gammopathies [22] we considered this aspect compatible with the elimination of free light chains. This suspicion was confirmed by cellulose acetate electrophoresis, which showed a homogeneous fraction of γ

mobility in the concentrated urine (fig 3) and by immunoelectrophoresis which confirmed the elimination of free Λ -chains in the urine (fig. 4) The total elimination of light chains was estimated to be 11.62 g/24 h based on the determination of total proteinuria and on the percentage determined for the light chain fraction by densitometry of the SDS-PAGE separation. A repeat study performed 12 months later showed the amount of Bence Jones protein to have decreased to 0.29 g/24 h. No abnormal fractions were detected in serum by either cellulose acetate electrophoresis or immunoelectrophoresis.

Patient II

The elimination of Bence-Jones protein was suspected, as in patient I because of the appearance of an abnormal fraction in the screening by SDS-PAGE (fig 2). The aspect was quite similar to that seen in patient I and the fraction of lowest molecular weight was estimated to represent 60% of the total eliminated protein by densitometry of the SDS-PAGE separation. The nature of this fraction was established by cellulose acetate electrophoresis, showing a homogeneous fraction of β -mobility in the concentrated urine (fig 3) and by immunoelectrophoresis, confirming the elimination of free Λ -chains in the urine (fig 4). The total Bence Jones proteinuria was estimated to be 0.59 g/24 h by the same type of calculation used for patient I. No abnormal fractions were seen in serum by either cellulose acetate electrophoresis or immunoelectrophoresis.

Discussion

Bence Jones proteinuria is classically considered as one of the hall marks of malignant plasma cell dyscrasias. It may be found associated with a monoclonal protein containing both heavy and light chains, representing an imbalance of the synthesis of light chains over heavy chains or it may also be an isolated event representing a more aberrant condition in which only light chains are synthesized or secreted [3-27]. In either case, Bence Jones proteinuria is thought to be an expression of biochemical de-differentiation and an index of malignancy [9-11].

An excess synthesis of light chains may occur in some cases otherwise classifiable as benign or idiopathic gammopathy [6, 13, 14]. Some of the reported observations of well-documented Bence Jones proteinuria in benign monoclonal gammopathy may be dismissed on the basis of short fol-

low-up. This point is well documented by HOMES [11] in his review on Bence Jones proteins, showing that in 76 cases of benign paraproteinemia, followed for more than 5 years, Bence Jones proteins were absent. However HOMES also mentions 75 cases of apparent benign character under observation, out of which 23 had Bence Jones proteins. It would be very unlikely that all the 23 turned out to be cases with malignant evolution.

The exclusive production and elimination of light chains are considered a very strong index of malignancy. Isolated Bence Jones proteinuria has been reported in 15% of patients with multiple myeloma [11] and with higher frequency in patients with invasion of soft tissues by the malignant plasma cells [11]. More rarely isolated Bence Jones proteinuria has been reported in patients with chronic lymphatic leukemia [23] and atypical plasma cell dyscrasia [4]. The finding of isolated Bence Jones proteins in an apparently healthy individual was reported for the first time by CAONSTEDT *et al.* [5] but this patient had antecedents of colonic carcinoma and evidence of immunodepression. A significant association of monoclonal gammopathies with neoplasias of the rectosigmoid colon has long been suspected, since their frequency is considerably higher in patients with colonic carcinoma than in any other group of cancer patients [12, 16]. The depression of immunoglobulin levels may also be considered as an indication of the probable malignant character of the plasma cell dyscrasia [9].

The association of monoclonal gammopathies with diabetes mellitus has never been recognized as significant. In a period of 12 years, only 8 cases of monoclonal gammopathy were observed among the diabetics treated at the Joslin Clinic [7]. Of the 2 cases reported in this paper at least 1 patient (patient II) seems to correspond to a true case of idiopathic Bence Jones proteinuria. The patient had no evidence of malignancy as opposed to patient I who had clear plasma cell infiltration of the bone marrow. However LINDSTRÖM and DAHLSTRÖM [14] have pointed out that bone marrow plasmocytosis can be found in patients with benign evolution. Precise classification of these 2 patients as cases of idiopathic Bence Jones proteinuria will be possible only after prolonged follow-up. Some authors have adopted an arbitrary minimum of 5 years of observation [10] but it is known that in some cases evolution to malignancy can take as long as 17 years [27]. We are continuing to screen diabetic patients to determine whether idiopathic Bence Jones proteinuria is more frequent in this group than in the population at large.

Acknowledgments

The authors wish to acknowledge the expert technical assistance of Mrs. WYON ANNE HIPP and Mr SHELTON ELLIS and the excellent editorial assistance of Mr CHARLES SMITH.

References

- 1 ALLANMITH, M. and CHANDOR, S. Asymptomatic monoclonal IgA hypergammaglobulinemia. *Am. J Med* 41 486-490 (1966)
- 2 AXELSON, U. BACHMANN R. and HALLEN J. Frequency of pathological proteins (M-components) in 6,995 sera from an adult population. *Acta med. scand.* 179 235-47 (1966)
- 3 BEVAN M. J. PARKHOUSE, R. M. J., WILLIAMSON A. J. and ASKONAS, H. A. Biosynthesis of immunoglobulins. *Prog. Biophys. molec. Biol.* 25 131-162 (1972)
- 4 CHENAI, F. VIRILLA, G. YOUNG, C. D. LIU P., and WITTE, G. T. Atypical B cell dyscrasia with Bence-Jones proteinuria and intracellular retention of γ -chains. *Acta haemat.* 58 166-177 (1977)
- 5 CROVSTEDT J. CARLINO, L., and OSTRERO H. Idiopathic light chain dyscrasia. A new distinct entity? *Acta med. scand.* 196 445-447 (1974).
- 6 DAMMACCO, F. and WALDENSTRÖM, J. Serum and urine light chain levels in benign monoclonal gammopathies, multiple myeloma and Waldenström's macroglobulinemia. *Clin. exp. Immunol.* 3 911-921 (1968)
- 7 GOLDSTEIN, H. H. Disorders of the blood. In MARBLE, WHITE, BRADLEY and KRALL Diabetes mellitus (Lea & Febiger Philadelphia 1971).
- 8 HALLEN J. Frequency of 'abnormal' serum globulins (M-components) in the aged. *Acta med. scand.* 173 737-744 (1963)
- 9 HOBBS, J. R. Paraproteins, benign or malignant? *Br med. J* iii 699-704 (1967)
- 10 HOBBS, J. R. Immunocytoma of Mice and Men *Br med J* ii 67-77 (1971)
- 11 HOBBS, J. R. Bence Jones proteins. *Essays med. Biochem.* 1 105-131 (1975)
- 12 ISORE, T. and OSTERMAN, E. F. Pathologic conditions associated with plasma cell dyscrasias: a study of 806 cases. *Ann. N. Y. Acad. Sci.* 190 507-518 (1971).
- 13 KYLF H. A., MALDONADO, J. E., and BAYRD, E. D. Idiopathic Bence Jones proteinuria. A distinct entity? *Am. J Med.* 55 222-226 (1973).
- 14 LINDSTRÖM, F. D. and DAHLSTRÖM, U. Multiple myeloma or benign monoclonal gammopathy (BMG)? A study of differential diagnostic criteria in 44 cases of BMG. *Clin. Immunology Immunopathol.* 10 168-174 (1978)
- 15 NORGÅRD O. Recherches sur l'évolution préclinique du myélome multiple. *Acta med. scand.* 176 137-146 (1964)
- 16 OSTERMAN, E. F. and FAHEY J. L. Plasma cell dyscrasias. Current clinical and biochemical concepts (combined staff clinic) *Am J Med.* 44 56 (1968).
- 17 OSTERMAN E. F. Plasma cell dyscrasia. In BEISON and McDERMOTT Textbook of medicine (Saunders, Philadelphia 1973)
- 18 PIER, M. T. CUNHA, A. S. VIRILLA, G. and SIMÕES, J. Analytical characterization of urinary proteins by sodium dodecyl sulphate-polyacrylamide gel electro-

- phorosis in renal disease. Clinical and histopathological correlations. *Nephron* 14 361-372 (1975).
- 19 RADL, J and MASOPUST J Idiopathische Paraproteinämie. *Schweiz. med. Wochr* 94 961-967 (1964).
- 20 RADL, J SEPPERS, J M., SEVARIH, F MORELL, A., and HUMANS, W Immunoglobulin patterns in human over 95 years of age. *Clin. exp. Immunol.* 22 84-90 (1975).
- 21 SOLOMONS, A. and FAHEY J L. Bence Jones proteinemia. *Am. J. Med.* 37 206-222 (1964).
- 22 VIRELLA, G. PIERA, M. T. and CORLINO, I. M. Analytical characterization of the urinary proteins from sixty patients with monoclonal gammopathies. *Clinica chim. Acta* 50: 63-73 (1975).
- 23 VIRELLA, G. SILVA, J J G. DA, LOPES-VIRELLA, M. F. L. y PARELLA, F. Cadenas lípidas intracelulares en los linfocitos periféricos de un enfermo con leucemia linfática crónica y proteinuria de Bence-Jones tipo lambda. *Sangre (Barcelona)* 20: 456-466 (1975).
- 4 WALDENSTRÖM, J. Abnormal proteins in myeloma. *Adv. internal Med.* 5 398-440 (1952).
- 25 WALDENSTRÖM, J. The occurrence of benign, essential monoclonal (M type) non-macromolecular hyperglobulinemia and its differential diagnosis. IV Studies in the gammopathies. *Acta med. scand.* 176 345-365 (1964).
- 26 WILLIAMS, R. C., BRIDGES, R. II and WOLLNEM, F. A.. Light chain disease. An abortive variant of multiple myeloma. *Ann. intern. Med.* 65 471-486 (1966).
- 27 ZOLA, S., BUXBAUM, J. FRANKLIN, E. C., and SCHARFF, M. D. Synthesis and assembly of immunoglobulins by malignant human plasmacytes. I. Myelomas producing γ -chains and light chains. *J. exp. Med.* 132 148-162 (1970).

Acknowledgments

The authors wish to acknowledge the expert technical assistance of Mrs. WENDY ANNE HIPP and Mr. SHELTON ELLIS and the excellent editorial assistance of Mr. CHARLES SMITH.

References

- 1 ALLANSMITH, M. and CHANDOR, S. Asymptomatic monoclonal IgA hypergammaglobulinemia. *Am. J. Med.* 41: 486-490 (1966).
- 2 AXELSSON, U. BACHMANN, R., and HALLEN, J. Frequency of pathological proteins (M-components) in 6,995 sera from an adult population. *Acta med. scand.* 179: 235-247 (1966).
- 3 BEVAN, M. J. PARKHOUSE, R. M. J., WILLIAMSON, A. J. and ASKONAS, B. A. Biosynthesis of immunoglobulins. *Prog. Biophys. molec. Biol.* 25: 131-162 (1972).
- 4 CHENAIS, F. VIRILLA, G., YOUNG, C. D. LIU, P. and WITTE, G. T. Atypical B cell dyscrasia with Bence-Jones proteinuria and intracellular retention of γ -chains. *Acta haemat.* 58: 166-172 (1977).
- 5 CRONSTEDT, J. CARLING, L., and OSTBERG, H. Idiopathic light chain dyscrasia. A new distinct entity? *Acta med. scand.* 196: 445-447 (1974).
- 6 DAMIACCO, F. and WALDENSTRÖM, J. Serum and urine light chain levels in benign monoclonal gammopathies, multiple myeloma and Waldenström's macroglobulinemia. *Clin. exp. Immunol.* 3: 911-921 (1968).
- 7 GOLDSTEIN, H. H. Disorders of the blood. In MARBLE, WHITE, BRADLEY and KRALL. *Diabetes mellitus* (Lee & Febiger Philadelphia 1971).
- 8 HALLEN, J. Frequency of abnormal serum globulins (M-components) in the aged. *Acta med. scand.* 173: 737-744 (1963).
- 9 HOBBS, J. R. Paraproteins, benign or malignant? *Br. med. J.* III: 699-704 (1967).
- 10 HOBBS, J. R. Immunocytoma of Mice and Men. *Br. med. J.* II: 67-72 (1971).
- 11 HOBBS, J. R. Bence Jones proteins. *Essays med. biochem.* 1: 105-131 (1975).
- 12 ISORE, T. and OSSERMAN, E. F. Pathologic conditions associated with plasma cell dyscrasias: a study of 806 cases. *Ann. N. Y. Acad. Sci.* 190: 507-518 (1971).
- 13 KYLE, R. A. MALDONADO, J. E., and BAYRD, E. D. Idiopathic Bence-Jones proteinuria. A distinct entity? *Am. J. Med.* 55: 222-226 (1973).
- 14 LINDBSTRÖM, F. D. and DAHLSTRÖM, U. Multiple myeloma or benign monoclonal gammopathy (BMG)? A study of differential diagnostic criteria in 44 cases of BMG. *Clin. Immunology Immunopathol.* 10: 168-174 (1978).
- 15 NORGAARD, D. Recherches sur l'évolution préclinique du myélome multiple. *Acta med. scand.* 176: 137-146 (1964).
- 16 OSSERMAN, E. F. and FAHEY, J. L. Plasma cell dyscrasias. Current clinical and biochemical concepts (combined staff clinic). *Am. J. Med.* 44: 256 (1968).
- 17 OSSERMAN, E. F. Plasma cell dyscrasia. In MELSON and McDERMOTT. *Textbook of medicine* (Sauders, Philadelphia 1975).
- 18 PIRER, M. T. CUNHA, A. S. VIRILLA, G. and SIMÕES, J. Analytic characterization of urinary proteins by sodium dodecyl sulphate-polyacrylamide gel electro-

11) Other 'lymphoid' features of CML-lyBC include biophysical and growth pattern characteristics [17] PAS-positivity [18] and the high content of deoxynucleotidyl-transferase [19]. Nevertheless, the agranular blasts have failed to prove their B or T nature by means of common surface markers [8-15]. Most recently JANOSSY *et al.* [8, 9] have reported that the blast cells of some cases of CML-lyBC strongly react with hetero-antisera specific for ALL-associated antigens, suggesting that acute lymphoblastic leukaemia (ALL) and CML-lyBC either involve the same aetiological agent or the same target cell [8].

In the present study antilymphocytic globulins (AHLGs), raised against both differentiated and transformed lymphoid cells, properly absorbed with myeloid and/or monocytic cells, and capable of discriminating between lymphoblastic and myeloblastic leukaemias [12] were reacted with peripheral blood cells from 12 cases of CML-BC. The strong reactivity of 1 case of the agranular type, measured by means of a microfluorimeter and compared to the weak fluorescence of the 11 'granular' CML-BC and other non-lymphoid leukaemias, the response to VCR PRED and the further transformation to an AHLG-non-reactive, VCR PRED refractory granular blastic crisis, warranted the present report.

Case Report

A 50-year-old female developed Ph-positive CML in December 1974 and was treated with busulfan and hydroxyurea until December 1975 when she was admitted to our Institution with severe pancytopenia, bone marrow particles at that time were not obtainable by means of needle aspiration. Intensive treatment with corticosteroids and transfusions allowed complete restoration of peripheral blood counts, and, after 1 month, hypercellular-regenerating bone marrow could be observed. The patient was allowed a period of rest, but after 2 weeks she was again admitted to the ward because of sudden drop in the haematocrit level to 18% with 200,000 WBC/l, and 1,000 platelets/l, differential counts showed 92% undifferentiated lymphoblast-like cells in the peripheral smear. The patient was treated with VCR mg on day 1 and with 6-methyl-prednisolone 125 mg/day for 2 days. After 48 h her WBC count dropped to 1,000/l with 40% neutrophils and 60% lymphocytes, and remission was maintained for 2 months with the same combination. In May 1976 the patient was again admitted to our Institution with 200,000 WBC/ μ L, Ht 40% and 800,000 platelets/l. Differential counts of peripheral blood smears showed 78% granular myeloblasts; the administration of VCR PRED was ineffective, so that the patient was given cytosine arabinoside and methotrexate, to which combination she responded for 8 months. In December 1976 she was admitted in coma to an other hospital and died 2 days later. Autopsy was not performed.

Lymphoid Antigens on Blast Cells in the Agranular Metamorphosis of Chronic Myelogenous Leukaemia¹

A. BACIGALUPO, G. SANTINI, G. PIAGGIO, M. RISSO, M. T. VAN LINT,
M. CARELLA, E. E. DAMASIO, M. C. RAFFO and A. M. MARMONT

Divisione di Ematologia ed Immunologia Clinica,
Ospedale Regionale S. Martino, Genova

Key Words: Chronic myelogenous leukaemia Antilymphocytic globulins
Leukaemia surface antigens

Abstract Undifferentiated blasts from a Ph positive chronic myelogenous leukaemia (CML) in terminal metamorphosis were reacted in an indirect immunofluorescence test with antilymphocytic globulins (AHLGs), raised against cultured lymphoblasts, thoracic duct and peripheral blood lymphocytes from healthy donors. After proper myeloid and/or monocytic absorptions the AHLGs interacted strongly with the undifferentiated blasts of CML, while this was not true for parallel controls with non lymphoid leukaemias, both acute and chronic. The intensity of fluorescence, as determined by the use of a microfluorimeter on these agranular blasts was comparable to the positivity of lymphoid cells from acute and chronic lymphatic leukaemias. These findings lend further support to the conception of a lymphoblast like variety of terminal blastic crisis in chronic myelogenous leukaemia.

The terminal blastic crisis of chronic myelogenous leukaemia (CML BC) can develop with two distinct morphologic features: the granular type with easily recognizable myeloblasts (CML myBC) and the agranular type with small blasts displaying lymphoblast like appearance (CML lyBC) [14]. Marked differences among these two subvarieties have been reported with regards to response to chemotherapy and lympholytic protocols: such as vincristine-prednisone (VCR PRED) have been shown to be more effective in the agranular type of CML BC [3].

Supported by Consiglio Nazionale delle Ricerche, CT 75 01230.04

11]. Other lymphoid features of CML-lyBC include biophysical and growth pattern characteristics [17] PAS-positivity [18] and the high content of deoxynucleotidyl-transferase [19]. Nevertheless, the agranular blasts have failed to prove their B or T nature by means of common surface markers [8, 15]. Most recently JANOSSY *et al* [8, 9] have reported that the blast cells of some cases of CML-lyBC strongly react with hetero-antisera specific for ALL associated antigens, suggesting that acute lymphoblastic leukaemia (ALL) and CML-lyBC either involve the same as biological agent or the same target cell [8].

In the present study antilymphocytic globulins (AHLGs), raised against both differentiated and transformed lymphoid cells, properly absorbed with myeloid and/or monocytic cells, and capable of discriminating between lymphoblastic and myeloblastic leukaemias [12] were reacted with peripheral blood cells from 12 cases of CML-BC. The strong reactivity of 1 case of the agranular type, measured by means of a microfluorimeter and compared to the weak fluorescence of the 11 granular CML-BC and other non-lymphoid leukaemias, the response to VCR-PRED and the further transformation to an AHLG-non-reactive, VCR-PRED refractory granular blastic crisis, warranted the present report.

Case Report

A 50-year-old female developed Ph-positive CML in December 1974 and was treated with busulfan and hydroxyurea until December 1975, when she was admitted to our Institution with severe pancytopenia, bone marrow particles at that time were not obtainable by means of needle aspiration. Intensive treatment with corticosteroids and transfusions allowed complete restoration of peripheral blood counts, and, after 1 month, hypercellular-regenerating bone marrow could be observed. The patient was allowed a period of rest, but after 2 weeks she was again admitted to the ward because of sudden drop in the haematocrit level to 18% with 200,000 WBC/ μ l, and 1,000 platelets/l. Differential counts showed 92% undifferentiated lymphoblast-like cells in the peripheral smear. The patient was treated with VCR 2 mg on day 1, and with 6-methyl-prednisolone 125 mg/day for 2 days. After 48 h her WBC count dropped to 1,000/ μ l with 40% neutrophils and 60% lymphocytes, and remission was maintained for 2 months with the same combination. In May 1976 the patient was again admitted to our Institution with 200,000 WBC/ μ l, Ht 40% and 800,000 platelets/l. Differential counts of peripheral blood smear showed 78% granular myeloblasts, the administration of VCR-PRED was ineffective, so that the patient was given cytotoxic arabinoside and methotrexate to which combination she responded for 8 months. In December 1976 she was admitted in coma to an other hospital and died 7 days later; autopsy was not performed.

Materials and Methods

Cell suspensions were obtained from 43 cases of acute and chronic leukaemias including CML BC (table I) separation of cells from EDTA-treated blood has been reported elsewhere [12]

AHLGs: Horse, anti-human lymphocytic globulins (AHLGs) raised against thoracic duct (TD) and peripheral blood lymphocytes (PB) from healthy donors and from lymphoblastoid cultures (L.L.) were obtained from Behringwerke, Marburg, already absorbed with erythrocytes and platelets. The AHLGs were composed of the gamma 1 fraction exclusively purification and absorption techniques have been described elsewhere [7]

Myeloid absorptions of AHLGs with cells from AML, CML and neutrophils from healthy donors were performed with 10^6 cells/2.5 mg of AHLG left for 1 h at 4 °C. Similar absorptions were carried out with monocytic cells from acute myelomonocytic (or monocytic) leukaemias. The procedures and effects of myeloid absorptions on AHLGs have been previously reported [12]

AHLGs reactions TD, PB, and L.L. AHLGs, after myeloid and/or monocytic absorptions, were reacted with a panel of leukaemic cell populations, comprising acute myeloblastic (AML), promyelocytic (APL), monocytic (AMoL), myelomonocytic (AMML) and chronic myelogenous leukaemias, besides acute and chronic lymphocytic leukaemias (ALL, CLL) and CML in blastic crisis (CML lyBC, CML-myBC), in an indirect immunofluorescence test, employing FITC-conjugated rabbit anti-horse immunoglobulin (RAHIF), as previously described [12]. Fluorescence was scored on 100 cells/slide as follows. (a) intensity of fluorescence - + + + + + + (b) percentage of fluorescent cells, and (c) pattern of fluorescence as spots, caps, or rings.

Microcytofluorimetry A Leitz microfluorimeter type MPW2, equipped with a photomultiplier EMI S20 and an HBO 75 W light, was employed to investigate membrane fluorescence after AHLG interaction and labelling of cells with RAHIF. The cells were focused in phase microscopy and the 'read' in epi-illumination \approx 570 nm, with a photomultiplier tension of 0.7 kV and a shutter of 0.25 sec. 50 cells were scored on every slide and statistical analysis was performed by calculating means, standard deviations, and 95% confidence limits. Control were performed as previously described [12]

Cytochemistry Cytochemical reactions were carried out on all cases and included PAS, peroxidase, Sudan black B, naphthol AS-D-acetate esterase, naphthol AS-D-chloroacetate esterase and α -naphthylacetate esterase.

Lymphocyte markers. Surface immunoglobulins (SIGs) were detected by reacting the cell suspensions with FITC-conjugated rabbit anti-human immunoglobulin (IgG fraction Cappel Laboratories) after the suspensions had been passed on Petri dishes for 30 min at 37 °C. E-rosettes were obtained by incubating the cell suspensions with sheep red blood cells (10^6 in saline) for 10 min at 37 °C the cells were then spun at 300 g for 10 min at 4 °C and subsequently kept at the same temperature for 2 h. After mounting the cells between slide and coverslip 200 cells/slide were scored phase microscopy

Results

Morphology and cytochemistry The morphological and cytochemical diagnosis of non-lymphoid and lymphoid acute leukaemias was performed according to current criteria. Of the 6 cases of ALL, all showed typical PAS-positivity. The one case of CML-lyBC showed 60% of blasts with coarse PAS-positivity while esterase reactions, Sudan black B and peroxidase were all negative. The same case, after developing a second blastic crisis, displayed Sudan black B peroxidase and esterase positivity (except for α -naphthylacetate esterase).

AHLG absorptions. AHLGs showed lymphoid specificity after proper absorptions with myeloid/monocytic cells (10^6 cells/2.5 mg of AHLG) for this study AHLGs were absorbed with 10^6 cells from AML or AMoL.

Lymphocyte markers. Of the 11 cases of ALL, 1 proved to be of B cell origin (Sig-positive), 1 of T-cell origin (E positive) and 4 were 'null' (Sig, E-negative). The agranular blastic crisis of CML did not show Sig-positive or E-rosetting blasts, therefore resembling null ALL.

AHLG reactivity After myeloid or monocytic absorptions AHLGs showed no further reactivity with cells from AML, CML, AMoL, AMML, APL, although a small percentage of cells were still positive and paralleled the percentage of lymphocytes in the cell suspension (table I). Lymphoid cells from ALL and CLL always displayed strong reactivity as well as B and T lymphocytes from healthy donors. Of the 12 cases of CML-BC, 11 (CML-myBC) were completely unstained, whereas in 1 case of CML-lyBC the reactivity of the leukaemic cell population could not be distinguished from the controls with ALL or CLL. It seems worthy to point out that also AHLGs elicited against mature lymphocytes (TD and PB AHLGs) reacted strongly with cells from CML-lyBC (table I fig. 2). The same case after remission-induction with VCR PRED developed a second blastic crisis with typical myeloblasts (fig. 3) in the peripheral smears, which proved to be AHLG-non reactive.

Microfluorimetric studies (fig. 4). These confirmed the data obtained by direct examination in immunofluorescence: acute and chronic non-lymphoid leukaemias scored counts in the range of 160 ± 47 while acute and chronic lymphocytic leukaemias scored counts in the range of 364 ± 24 the differences between lymphoid and non-lymphoid leukaemias were statistically significant ($p < 0.01$). Occasionally lymphocytes with high counts could be observed in AML and AMoL and were

Materials and Methods

Cell suspensions were obtained from 43 cases of acute and chronic leukaemias including CML BC (table I) separation of cells from EDTA-treated blood has been reported elsewhere [12]

AHLGs Horse, anti-human lymphocytic globulins (AHLGs) raised against thoracic duct (TD) and peripheral blood lymphocytes (PB) from healthy donors and from lymphoblastoid cultures (KL), were obtained from Behringwerke, Marburg, already absorbed with erythrocytes and platelets. The AHLGs were composed of the gamma 1 fraction exclusively: purification and absorption techniques have been described elsewhere [7]

Myeloid absorptions of AHLGs with cells from AML, CML and neutrophils from healthy donors were performed with 10^5 cells/2.5 mg of AHLG left for 1 h at 4 °C. Similar absorptions were carried out with monocytic cells from acute myelomonocytic (or monocytic) leukaemias. The procedures and effects of myeloid absorptions on AHLGs have been previously reported [12].

AHLGs reactions. TD PB and KL AHLGs, after myeloid and/or monocytic absorptions, were reacted with a panel of leukaemic cell populations, comprising acute myeloblastic (AML), promyelocytic (APL), monocytic (AMoL), myelomonocytic (AMML) and chronic myelogenous leukaemias, besides acute and chronic lymphocytic leukaemias (ALL, CLL) and CML in blastic crisis (CML-lyBC, CML-myBC), in an indirect immunofluorescence test, employing FITC-conjugated rabbit anti-horse immunoglobulin (RAHIF) as previously described [1]. Fluorescence was scored on 100 cells/slide as follows (a) intensity of fluorescence, -, +, ++, +++ (b) percentage of fluorescent cells, and (c) pattern of fluorescence as spots, caps, or rings.

Microcytofluorimetry A Leitz microfluorimeter type MPW2, equipped with a photomultiplier EMI S20 and an HBO 75 W light, was employed to investigate membrane fluorescence after AHLG interaction and labelling of cells with RAHIF. The cells were focused in phase microscopy and the 'read' in epi-illumination at 520 nm, with a photomultiplier tension of 0.7 kV and a shutter of 0.25 sec. 50 cells were scored on every slide, and statistical analysis was performed by calculating means, standard deviations, and 95% confidence limits. Controls were performed as previously described [12].

Cytochemistry Cytochemical reactions were carried out on all cases and included PAS, peroxidase, Sudan black B, naphthol AS-D-acetate esterase, naphthol AS-D-chloroacetate esterase and α -naphthylacetate esterase.

Lymphocyte markers Surface immunoglobulins (SIGs) were detected by reacting the cell suspensions with FITC-conjugated rabbit anti-human immunoglobulin (IgG fraction, Cappel Laboratories) after the suspensions had been passed on Petri dishes for 30 min at 37 °C. E rosettes were obtained by incubating the cell suspensions with sheep red blood cells (10% in saline) for 10 min at 37 °C the cells were then spun at 300 g for 10 min at 4 °C and subsequently kept at the same temperature for 2 h. After mounting the cells between slide and coverslip, 200 cells/slide were scored phase microscopy

Table 1 Cells reacting with myeloid-monocytic absorbed AHLGs. Intensity of fluorescence (int. direct observation), percentage of fluorescent cells (%) and pattern (part.) of fluorescence

	Cases	KL-AHLG			PB-AHLG			TD-AHLG		
		int.	%	part.	int.	%	part.	int.	%	part.
AML	6	+	8	spots	++	9	ring	++	7	ring
APL	2	+	2	spots	++	4	ring	++	5	ring
AMoL	3	+	4	spots	++	3	spots	++	4	spots
AMML	2	+	5	ring	++	8	ring	++	5	ring
CML	4	+	1	spots	++	16	ring	++	15	ring
CML-myBC	11	+	8	spots	++	7	ring	++	7	ring
CML-lyBC	1	+++	90	ring	+++	92	ring	+++	89	ring
ALL (T/B/both)	6	+++	95	ring	+++	90	ring	+++	92	ring
CLL	8	+++	98	ring	+++	95	ring	+++	98	ring
T/B Lymph.	3	+++	99	ring	+++	97	ring	+++	95	ring
Neutrophils	3	-			-			-		

AML = Acute myelogenous leukaemia, APL = acute promyelocytic leukaemia, AMoL = acute monocytic leukaemia, AMML = acute myelomonocytic leukaemia, CML = chronic myelogenous leukaemia, CML-myBC = CML in 'myeloid' blastic crisis, CML-lyBC = CML in 'lymphoid' or agranular blastic crisis, ALL = acute lymphoblastic leukaemia (T/B/both), CLL = chronic lymphocytic leukaemia, T/B/ Lymph. and neutrophils from 3 healthy donors

not considered in the final score. In the CML-lyBC, high counts, 347 ± 17 could be obtained on 2 consecutive days, employing both TC, PB and KL AHLGs, there was no statistical difference with lymphoid leukaemias, whereas AML and AMoL scored low counts (158 ± 12 and 167 ± 15 respectively) on the same occasion. Microfluorimetric studies of the same case after the development of a granular metamorphosis (fig. 3) showed low scores in the range of myeloid leukaemias (141 ± 11).

Fig. 2 Blasts from CML-lyBC reacted in indirect immunofluorescence with MY monocytic absorbed TD-AHLG. Ring type fluorescence of two large blasts and of smaller 'lymphocyte-like' cell. 800.

Fig. 3 Blasts from the CML-lyBC after relapse from VCR/PRED induced remission (third phase of the disease). The morphology of the cells is that of typical 'granular' myeloblasts (CML-myBC). $\times 800$.

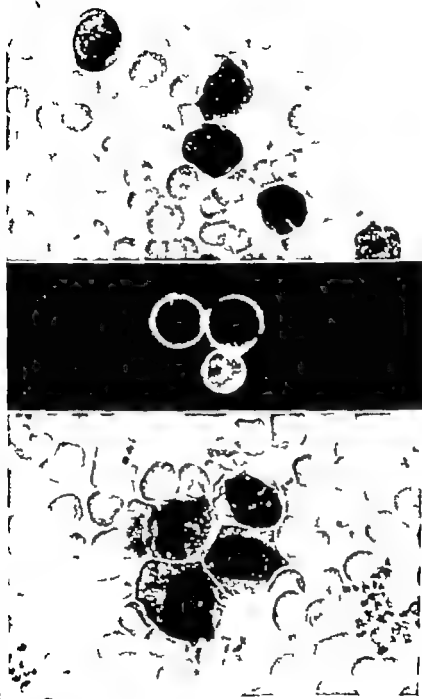


Fig 1 Peripheral blood smears of the case reported (CML lyBC) stained according to Romanowsky Agranula blasts with scanty poorly stained cytoplasm can be seen some nuclei are cleaved $\times 800$

Discussion

The 'agranular' lymphoblast-like metamorphosis of CML has recently received much attention because of its striking similarities with common (non-T non-B) ALL. JANOSSY *et al.* [8] have investigated the reactivity of CML-lyBC to an anti ALL antiserum, specific for ALL-associated antigens, and the resulting data would suggest that ALL and CML-lyBC share common antigenic determinants. the possible explanation for this finding would be either a common aetiological agent (virus?) or the involvement of closely related target cells [8]. However it must be pointed out that anti-ALL antisera as those employed in the above mentioned and other studies [2, 9, 10, 16] are claimed to be directed against leukaemia-associated antigens and, because of proper absorptions with peripheral blood lymphocytes, do not react with B or T cells. In other words ALL antisera should be devoid of antibodies capable of recognizing differentiation antigens expressed on both mature and immature lymphoid (LY) cells, but only leukaemia associated antigens or other determinants, restricted to that particular stage of differentiation, should react with such antisera. Therefore the cross-reactivity of ALL antisera with other cell populations, and particularly with CML-lyBC, though providing new aetiological perspectives, would not allow but indirect speculations on the origin of the reactive cells.

In the present report we have shown that AHLGs, elicited against mature (TD PB) and transformed (KL) lymphoid cells, after appropriate absorptions with myeloid (MY) cells, can still interact with peripheral blood lymphocytes (both T and B cells) from healthy donors, lymphocytes from CLL, blasts from ALL and with the lymphoid-like cell population of CML-lyBC. Cells from CML-myBC and from AML, AMoL, AMML, APL and CML as well as neutrophils from healthy donors no longer reacted with MY-absorbed AHLGs: these observations (table I) were confirmed by microcytofluorimetry (fig. 4) and statistical analysis proved the LY-MY gradient to be highly significant ($p < 0.01$). Furthermore cells from CML-lyBC were capable of absorbing anti-lymphoid antibodies out of TD PB and KL AHLGs, thus reducing CLL and ALL counts down to levels where the LY-MY gradient was no longer significant (fig. 5). AHLGs were employed with the specific goal of recognizing an antigenic complex, an LY complex, possibly expressed all along the lymphoid differentiation pathway on both thymus and bone marrow derived cells, so that B/T absorptions were purposely avoided. The strong reactivity of all

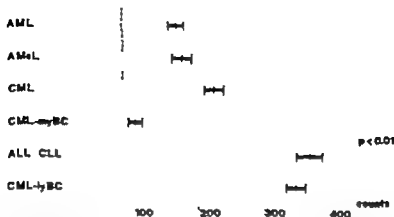


Fig 4 Microfluorimetry of leukaemic cells interacting with myeloid absorbed TD-AHLG: differences between lymphoid and non-lymphoid leukaemias are statistically significant ($p < 0.01$). AML = 158 ± 17 AMoL = 167 ± 15 CML = 17 ± 12 CML myBC = 100 ± 11 ALL-CLL (mean) = 364 ± 24 CML IyBC = 347 ± 17 $\pm = 95\%$ confidence limits.

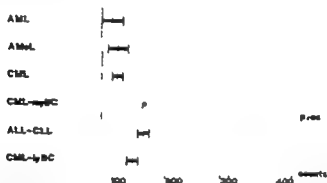


Fig 5 Microfluorimetry of leukaemic cells interacting with TD-AHLG after absorption with myeloid cells and with the agranular blasts from CML IyBC. Scores of both acute and chronic lymphatic leukaemias as well as CML IyBC are markedly reduced. The AHLG is no longer discriminating among the latter and non-lymphoid leukaemias. AML = 100 ± 18 AMoL = 111 ± 19 CML = 108 ± 9 ALL = 158 ± 12 CML-IyBC = 141 ± 11

Absorption of AHLG with the granular blasts of CML IyBC This produced a reduction of membrane fluorescence in lymphoid leukaemias, and microfluorimetric studies (fig 5) showed that the antisera were no longer discriminating lymphoid from myeloid leukaemias.

Discussion

The 'agranular' lymphoblast like metamorphosis of CML has recently received much attention because of its striking similarities with common (non-T non-B) ALL. JANOSSY *et al* [8] have investigated the reactivity of CML-lyBC to an anti ALL antiserum, specific for ALL-associated antigens, and the resulting data would suggest that ALL and CML lyBC share common antigenic determinants. the possible explanation for this finding would be either a common aetiological agent (virus?) or the involvement of closely related target cells [8]. However it must be pointed out that anti-ALL antisera as those employed in the above mentioned and other studies [2, 9, 10, 16] are claimed to be directed against leukaemia-associated antigens and, because of proper absorptions with peripheral blood lymphocytes, do not react with B or T cells. In other words ALL antisera should be devoid of antibodies capable of recognizing differentiation antigens expressed on both mature and immature lymphoid (LY) cells, but only leukaemia-associated antigens or other determinants, restricted to that particular stage of differentiation, should react with such antisera. Therefore the cross-reactivity of ALL antisera with other cell populations, and particularly with CML lyBC, though providing new aetiological perspectives, would not allow but indirect speculations on the origin of the reactive cells.

In the present report we have shown that AHLGs, elicited against mature (TD PB) and transformed (KL) lymphoid cells, after appropriate absorptions with myeloid (MY) cells, can still interact with peripheral blood lymphocytes (both T and B cells) from healthy donors, lymphocytes from CLL, blasts from ALL and with the lymphoid-like cell population of CML lyBC. Cells from CML-myBC and from AML, AMoL, ANML, APL and CML as well as neutrophils from healthy donors no longer reacted with MY-absorbed AHLGs: these observations (table I) were confirmed by microcytofluorimetry (fig. 4) and statistical analysis proved the LY MY gradient to be highly significant ($p < 0.01$). Furthermore cells from CML-lyBC were capable of absorbing anti-lymphoid antibodies out of TD PB and KL AHLGs, thus reducing CLL and ALL counts down to levels where the LY MY gradient was no longer significant (fig. 5). AHLGs were employed with the specific goal of recognizing an antigenic complex, an LY complex, possibly expressed all along the lymphoid differentiation pathway on both thymus and bone marrow derived cells, so that B/T absorptions were purposely avoided. The strong reactivity of all

ALL, irrespectively of their T II or null characteristics, of CLL and of mature B/T lymphocytes to MY absorbed AHLGs would confirm our working hypothesis, suggesting that the LY complex can be expressed on the surface of B/T lymphocytes and also on leukaemic cell populations [12-13] in agreement with the data of JANOSSY *et al* [9]

In the case of Ph positive CML here reported *in extenso* three different phases can be recognized: in the first the classical peripheral blood cell population of CML was Ph positive and AHLG non reactive; in the second, the agranular blasts (lymphoblast like blasts) showed strong reactivity to MY absorbed AHLGs and proved to be VCR PRED sensitive; the Ph-chromosome not being detected because no analyzable metaphases could be obtained; finally after relapse from remission induction with VCR PRED in the third phase of the disease the myeloblastic cell population was again AHLG-non-reactive Ph positive and VCR PRED refractory.

The most relevant finding of this study is the *strong* reactivity of CML lyBC to AHLGs raised against thoracic duct or peripheral blood lymphocytes as well as against transformed LY cells, whereas JANOSSY *et al* [9] have reported a *weak* positivity of CML lyBC to AHLGs raised against tonsil lymphocytes in 2 cases and the absence of reactivity in 3 cases.

The recognition of LY antigens on the surface of agranular blasts in CML raises the question of the target cell of the Ph abnormality: the variability of blastic transformation in CML, comprising myeloid, lymphoid, monocytic and possibly erythroid and megakaryocytic subvarieties, as well as the concept of a spectrum of lymphoid-myeloid expression for Ph positive childhood leukaemias [1-5] would favour the involvement of a pluripotent haemopoietic stem cell, capable of differentiation along all the bone marrow derived cell lines. However the expression of LY antigens on cells from CML lyBC does not necessarily prove their lymphoid origin since both stem cells [4-6] and acute undifferentiated leukaemias [13] have been shown to be cross reacting with AHLGs. One can indeed prove that CML lyBC as well as common (non T non B)-PAS negative ALL, are not committed along the myeloid differentiation pathway because they do not react to anti myeloid antisera [unpublished data] but it is difficult to ascertain whether these cells are 'stem cells' cross reacting with AHLGs or non T non B lymphoblasts. Actually it is not known whether such a distinction does exist at all and what is called an 'uncommitted lymphoblast' could indeed be a stem cell.

Although at present it is difficult to assess the real nature of AHLG-reactive blasts (stem cells, uncommitted lymphoblasts?), the expression of LY antigens seems to be somehow linked to VCR-PRED responsiveness, and this is true for CML-lyBC, ALL, as well as for acute undifferentiated leukaemias [unpublished observations].

Our data would thus confirm and lend further support to the hypothesis that CML is a disease arising in a pluripotent stem cell and that the agranular blasts of CML-lyBC do express LY antigens on the cell surface. Nevertheless, more work with anti-myeloid antisera and with 'physiologic stem cells' is needed in order to establish the relationship between levels of stem cell differentiation and expression of cell line specific antigens.

Acknowledgements

We wish to thank Dr G. NICCOLÒ, Dr G. GRADONIA and Dr M. FURTONI of the Institute of Oncology University of Genova (Director Prof. L. SANTI) for providing the microcytofluorimeter and for constructive advice in the elaboration of the resulting data.

References

- 1 BOON, D. R. Haemopoietic stem cell theory in relation to possible lymphoblastic conversion of chronic myeloid leukaemia. *Blood* 44: 449-453 (1974).
- 2 BROWN, G., HOOD, N., and GRADY, M. III. Candidate leukaemia-specific antigens in man. *Nature*, Lond. 258: 454-456 (1975).
- 3 CAMELLO, G. P., VITA, V. T. DE, WILANO-PINO, J., CHAMBER, B. A., SCHEN, P. S. and YOUNG, R. C. Chemotherapy of the blastic phase of chronic granulocytic leukaemia. hypodiploidy and response to therapy. *Blood* 47: 1003-1009 (1976).
- 4 FIELD, E. O. and GIBBS, J. E. Cross-reaction of antilymphocyte serum with haemopoietic stem cells. *Nature*, Lond. 217: 561-562 (1968).
- 5 FORMAN, E. N., PADRÒ-MENDOZA, T., SMITH, P. S., BARKER, B. E., and FARBER, I. III. Ph⁺-positive childhood leukaemia. Spectrum of lymphoid-myeloid expression. *Blood* 49: 549-558 (1977).
- 6 GOLUB, E. Brain associated cell antigen: an antigen shared by brain and haemopoietic stem cells. *J. exp. Med.* 136: 369-374 (1972).
- 7 HEIDE, K. and SEILER, F. R. Purification of ALG⁺ problems with the isolation of the relevant antibodies only. *Behr. Int. Mik.* 51: 93-95 (1972).
- 8 JAMESON, G., ROBERTS, M., and GRADY, M. F. Target cell in chronic myeloid leukaemia and its relationship to acute lymphoid leukaemia. *Lancet* ii: 1058-1060 (1976).

ALL, irrespectively of their T II or null characteristics, of CLL and of mature B/T lymphocytes to MY absorbed AHLGs would confirm our working hypothesis, suggesting that the LY complex can be expressed on the surface of B/T lymphocytes and also on leukaemic cell populations [12-13] in agreement with the data of JANOSSY *et al* [9].

In the case of Ph positive CML here reported *in extenso* three different phases can be recognized: in the first the classical peripheral blood cell population of CML was Ph-positive and AHLG non reactive; in the second, the agranular blasts (lymphoblast like blasts) showed strong reactivity to MY-absorbed AHLGs and proved to be VCR PRED sensitive; the Ph-chromosome not being detected because no analyzable metaphases could be obtained; finally after relapse from remission induction with VCR PRED in the third phase of the disease the myeloblastic cell population was again AHLG non reactive Ph positive and VCR PRED refractory.

The most relevant finding of this study is the *strong* reactivity of CML-lyBC to AHLGs raised against thoracic duct or peripheral blood lymphocytes as well as against transformed LY cells, whereas JANOSSY *et al* [9] have reported a *weak* positivity of CML lyBC to AHLGs raised against tonsil lymphocytes in 2 cases and the absence of reactivity in 3 cases.

The recognition of LY antigens on the surface of agranular blasts in CML raises the question of the target cell of the Ph abnormality: the variability of blastic transformation in CML, comprising myeloid lymphoid, monocytic and possibly erythroid and megakaryocytic subvarieties, as well as the concept of a spectrum of lymphoid myeloid expression for Ph positive childhood leukaemias [1-5] would favour the involvement of a pluripotent haemopoietic stem cell capable of differentiation along all the bone marrow derived cell lines. However the expression of LY antigens on cells from CML lyBC does not necessarily prove their lymphoid origin since both stem cells [4-6] and acute undifferentiated leukaemias [13] have been shown to be cross-reacting with AHLGs. One can indeed prove that CML-lyBC as well as common (non T non B)-PAS negative ALL, are not committed along the myeloid differentiation pathway because they do not react to anti myeloid antisera [unpublished data] but it is difficult to ascertain whether these cells are stem cells cross-reacting with AHLGs or non-T non-B lymphoblasts. Actually it is not known whether such a distinction does exist at all and what is called an 'uncommitted lymphoblast' could indeed be a stem cell.

Defect of Bone Marrow Granulocyte Reserve in Liver Cirrhosis Evaluated with Etiocholanolone

G. CAMBIAGHI and S. PAINA

Department of Internal Medicine (Chief: Prof. III C. Secchi),
Istituto Clinico di Perfezionamento, Milano

Key Words. Cirrhosis · Bone marrow · Granulocyte reserve · Etiocholanolone

Abstract. In patients with cirrhosis, the etiocholanolone test showed a decrease in the bone marrow granulocyte reserve; in all the cases studied, the baseline counts of peripheral granulocytes were normal. The mechanisms leading to such defect are explained.

Several authors have shown that the marrow granulocyte reserve can be studied by the peripheral granulocyte response to injection of pyrogens [1-2], etiocholanolone [3-6] and cortisone [7].

These tests could be very useful for studying early stages of bone marrow failure, when peripheral neutropenia is still absent. Using the etiocholanolone test, we studied the bone marrow reserve of granulocytes in patients suffering from liver cirrhosis and observed a significant decrease of the peripheral granulocyte response to injection of etiocholanolone.

Materials and Methods

Patients. A total of 17 patients were studied, 11 males and 6 females, aged 35-72 years, suffering from advanced liver cirrhosis. All patients had portal hypertension (esophageal varices and slight splenomegaly).

The diagnosis of cirrhosis was based on history, clinical signs and symptoms and on the following laboratory findings: serum proteins electrophoresis, IgA-IgM IgG

- 9 JANOSY G GREAVES, M. F. REVEZ, T. LISTER, T. A. ROBERTS, M. DURRANT J. KIRK, B., CATOVSKY D. and BEARD M. E.. Blast crisis of chronic myeloid leukaemia (CML) II. Cell surface marker analysis of lymphoid and myeloid cases. *Br J Haemat* 34 179-192 (1976)
- 10 MANN, D. L. HALTERMAN, R., and LEVENTHAL, B.. Acute leukaemia associated antigens. *Cancer* 34 suppl. pp. 1446-1451 (1974).
- 11 MARMONT A. M. and DAMASIO, E. E.. The treatment of terminal metamorphosis of chronic granulocytic leukaemia with corticosteroids and vincristine. *Acta haemat* 51 1-8 (1973).
- 12 MARMONT A. M. SANTINI, G., BACIGALUPO, A. DAMASIO, E. E., and GIORDANO, D.. Immunofluorescent diagnosis of acute lymphoblastic leukaemia. *Acta haemat* 54 129-142 (1975)
- 13 MARMONT A. M. SANTINI G. BACIGALUPO, A., and DAMASIO, E. E. Immunocytological investigations in the apparently undifferentiated acute leukaemias; in STACHUR und HOCKER Erkrankungen der Myelopoese, pp 171-173 (Urban & Schwarzenberg, Wien 1976)
- 14 MATHÉ, G. et SEMAN G. Aspects histologiques et cytologiques des leucémies et hématosarcomes, vol. 1 p. 22 (Maloine, Paris 1963).
- 15 MATHÉ, G., BELPOMER, D., DANTCHEV D., FOULLART P. NAVARIS, L. HAUS, G., SCHLIMBERG J. R., and LAPLEUR, M.. Search for correlations between cytological types and therapeutic sensitivity of acute leukaemias. *Blood Cells* 1 37-41 (1975)
- 16 METZGAR, R. S., MOHAMMADUMAR, T., and MILLER, D. S.. Antigens specific for human lymphocytic and myeloid leukaemia cells: detection by non-human primate antisera. *Science* 178 986-988 (1972).
- 17 MOORE, M. A. S. Marrow culture: a new approach to classification of leukaemias. *Blood Cells* 1 149-155 (1975)
- 18 PEDERSEN, B.. Clonal evolution and progression in chronic myeloid leukaemia. *Blood Cells* 1 227-230 (1975).
- 19 SARDI, P. S. ANDERSON P. N. and GALLO, R. C.. Terminal deoxynucleotidyl transferase activities in human blood leukocytes and in lymphoblast cell lines: high levels in lymphoblast cell lines and in blast cells of some patients with chronic myelogenous leukaemia in the acute phase. *Blood* 47 11-20 (1976).

Table I. Changes in bone marrow reserve in 10 healthy controls and in 17 cirrhotic subjects

	Baseline counts of granulocytes/ μ l	Granulocytes/ μ l			ΔG
		after 9 h	after 12 h	after 15 h	
<i>Healthy controls</i>					
1	5,500	6,200	8,900	7,900	3,400
2	5,000	5,900	7,900	7,600	2,900
3	5,100	7,000	9,100	8,800	4,000
4	5,200	7,100	8,200	7,800	3,000
5	5,900	8,000	10,000	9,100	4,100
6	4,900	6,200	7,900	7,000	3,000
7	5,500	8,200	9,700	9,000	4,200
8	5,200	6,100	8,200	7,800	3,000
9	5,000	7,100	8,600	8,100	3,600
10	5,600	8,600	10,100	9,100	4,500
Mean	5,290				3,570
SD	± 521				± 594
CV	6.06				16.6
<i>Cirrhotic subjects</i>					
1	3,900	4,800	5,300	5,000	2,000
2	4,900	5,100	6,000	5,600	1,900
3	5,800	6,200	6,600	6,300	800
4	4,700	5,000	5,900	5,500	1,200
5	5,100	5,700	6,100	5,800	1,000
6	6,000	6,500	6,900	6,600	900
7	4,900	6,400	6,900	6,300	2,000
8	5,900	5,600	5,800	5,500	300
9	9,000	10,600	11,300	11,000	2,300
10	4,900	4,700	5,000	4,800	900
11	5,000	5,000	5,100	5,000	100
12	5,900	6,100	6,400	6,200	400
13	3,900	4,100	4,300	4,200	600
14	4,000	4,000	4,100	4,000	100
15	5,100	6,900	7,000	6,800	1,900
16	3,800	4,100	5,000	4,800	1,200
17	4,600	4,600	4,700	4,600	100
Mean	5,076				994
SD	$\pm 1,235$				± 731
CV	4.3				73.5

dosage, prothrombinemia, pseudocholinesterase, SGOT and SGPT levels, HbAg, total serum bilirubin, BSF clearance, serum γ -GT and alkaline phosphatase. In all patients, the diagnosis was confirmed by liver needle biopsy.

Care was taken to exclude from this study all patients whose conditions could modify the granulocyte response (bacterial infections, simultaneous treatment with corticoids, etc.) All patients studied were absolutely afebrile. A group of 10 healthy controls, 5 males and 5 females, was also studied after careful screening of their clinical conditions. Both the patients and the controls were fully informed about the studies that would be done and their agreement was obtained.

Etiocolanolone test The method of Vogel *et al.* [3] was followed. Exactly at midnight each individual received an intramuscular injection of 0.1 mg/kg of a 1% solution of etiocolanolone in propylene glycol. At 3 p.m. on the previous day and 9, 12 and 15 h after the injection, peripheral granulocyte counts were performed on duplicates in a Bürker chamber. The differential count was determined before and after the test by counting at least 200 cells in smears stained according to the May Grünwald-Giemsa method.

The difference between the baseline count and the maximum granulocyte count obtained at any hour following the injection of etiocolanolone (ΔG) was calculated.

Results

The results are summarized in table I which shows the individual data, the means with standard deviations (SD) and the coefficient of variation (CV) of the baseline counts of granulocytes and of ΔG in healthy controls and in cirrhotic subjects. In healthy controls, the mean of baseline counts of granulocytes is $5,290 \pm 321/\mu l$ the CV is 6.06. In cirrhotic patients, the mean of baseline counts of granulocytes is $5,076 \pm 1,235/\mu l$, the CV is 24.3. These baseline counts did not differ significantly ($t = 2.58$, $p = 0.02$). In all 17 patients, the bone marrow granulocyte response after stimulation was decreased or absent. In healthy controls, the average value of ΔG is $3,570 \pm 594/\mu l$ and the CV is 16.6. In cirrhotic patients the average value of ΔG is $994 \pm 731/\mu l$ and the CV is 73.5. Though the results vary widely the average value of ΔG observed in the patients suffering from cirrhosis, is significantly reduced compared to the average ΔG value observed in the healthy controls ($t = 9.45$, $p = 0.0005$).

Discussion

The etiocolanolone test showed that in patients suffering from cirrhosis with a normal baseline count of peripheral granulocytes the bone mar-

- 8 FIESCHI, A., SACCHETTI, C., Aspetti di fisiopatologia clinica dell'ipersplenismo. Proc. 68° Congr. Soc. Ital. Med. Interna, Firenze 1967
- 9 MACIUSO, E., GALLIGA, M., SCEVOLO, D., and COSCIA, E., Defect of bone marrow granulocyte reserve in viral hepatitis. *Acta haemat.* 54: 27-31 (1975).

row reserve is reduced. The mechanisms leading to such a defect are not easy to explain. They might result from a myelotoxic effect consequent to lack of proteins and vitamins, induced by cirrhosis. Moreover in liver cirrhosis, especially in the late stage a concomitant hypersplenism consequent to spleen enlargement is always present. The increased sequestration and/or waste of granulocytes by the spleen induces a compensative hyperproduction of these cells by the bone marrow [8] the bone marrow activity can be sufficient to maintain a normal number of circulating granulocytes, but not to maintain a normal bone marrow reserve.

Finally in some cases of cirrhosis, an important pathogenetic role can be played by past viral hepatitis, and a correlation between hepatitis and bone marrow damage has, on the other hand, already been reported [9]. It therefore seems that the etiocholanolone test might be important in detecting an early bone marrow impairment in patients with cirrhosis, and might hence provide findings useful for the prognostic evaluation of this disease.

References

- 1 FINK, E. M. and CALABRESI, P. The granulocyte response to an endotoxin (Pyrexal) as a measure of functional marrow reserve in cancer chemotherapy. *Ann. Intern. Med.* 57: 732-742 (1962).
- 2 MECHANIC, R. C. and FRIE, E. Human leucocyte and febrile response to graduated and repeated doses of a purified bacterial endotoxin. *Clin. Res.* 9: 163 (1961).
- 3 VOGEL, J. M., GARHEE, R. A., KIMBALL, H. R., WOLFF, S. M., and PERRY, S. The effect of etiocholanolone on granulocyte kinetics. *Blood* 30: 474-483 (1967).
- 4 KIMBALL, H. R., VOGEL, J. M., PERRY, S., and WOLFF, S. M. Quantitative aspects of pyrogenic and haematologic response to etiocholanolone in man. *J. Lab. clin. Med.* 69: 415-427 (1967).
- 5 VOGEL, J. M., KIMBALL, R. H., WOLFF, S. M. and PERRY, S. Etiocholanolone in the evaluation of marrow reserves in patients receiving cytotoxic agents. *Ann. Intern. Med.* 67: 1226-1238 (1967).
- 6 KARJALAINEN, J. and WARASTIERMA, C. The etiocholanolone test for prediction of the leukopenic effect of cytotoxic drugs. *Scand. J. Haematol.* 11: 337-341 (1973).
- 7 DALK, D. C., FAUCI, A. S., GUERRY, D. and WOLFF, S. M. Comparison of agents producing a neutrophilic leukocytosis in man. Hydrocortisone, prednisone, endotoxin and etiocholanolone. *J. clin. Invest.* 56: 803-813 (1975).

Materials and Methods

Dialysis in Visking casing [Z] was performed for 24 h at 4 °C against two standard 0.1 M phosphate buffers of pH 7 and pH 3. pH monitoring showed that 0.5–1.5 ml test plasmas attained the pH of the buffer within 2 h.

Two radioactive isotopes used, were obtained from the Radiochemical Centre, Amersham, UK. (i) folic acid (potassium salt), labelled with ^3H in the 3' 5' 9' positions in the phenyl group (^3H -PteGlu), molecular mass 441, spec. act. 5 Ci/mmol, radiochemical purity 98%, and (ii) 5 (methyl ^{14}C)-tetrahydrofolic acid (barium salt) (^{14}C -5CH₃H₄PteGlu), molecular mass 666, spec. act. 50–60 mCi/mmol, radiochemical purity 99%. The salts were dissolved in sodium hydroxide and ethanol as in preparing standard folic acid curves for the *Lactobacillus casei* biofolate assay of Himmelfarb [11], dried ascorbic acid (1 mg/ml) added to methylfolate and stored in the dark at -20 °C. Solutions were bioassayed to ensure that biofolate content corresponded to calculated radiofolate concentrations.

Radioactivity was measured by Liquid scintillation counting on an Lister technique SL 30 connected to Multis-9 computer [12].

The *in vitro* folate-binding capacity of plasma was measured after incubation with excess ^{14}C -5CH₃H₄PteGlu and ^3H PteGlu added simultaneously as previously described [12], except that free radiofolate was removed by dialysis and not by charcoal adsorption.

Liquid chromatography with Sephadex DEAE-A50- 20 × 0.8 cm columns were used, 0.5 ml samples applied, and 40–50 × 1 ml fractions eluted stepwise with 7 × 5 ml buffer NaCl solutions at pH 7 [12]. Eluates were measured for radioactivity and total protein (UV extinction at 280 nm); eluates 0–10 represent γ -globulins and fibrinogen, 10–16 β -globulin with transferrin, 16–23 α -globulins, 25–45 albumin and prealbumin. The elution pattern for radiofolate standards in this system (whether applied in plasma or 0.9% NaCl) is as follows: peak elution for PteGlu at eluates 39–45, for 5 CH₃H₄PteGlu at eluates 30–36.

Plasma in heparinized 'Vacutainer' tubes (Beckton-Dickinson) were collected from (i) 4 normal subjects, and (ii) 2 subjects with normal folate status in whom the endogenous binder had been labelled *in vivo* after ingestion of radiofolate, as previously described [8, 13]. Subjects gave informed consent for this study which had been accepted by the Medical Faculty's Ethical Research Committee and the Radiation Control Committee for the Orange Free State (acting on behalf of the Atomic Energy Board). 3 subjects (with normal folate status) took 575 μg ^{14}C -CH₃H PteGlu (50 μCi). Heparinized blood was taken at 6 h (subject 1, III) or 24 h (subject 3) after isotope absorption, and plasma stored at -20 °C.

Results

In vitro Binding

The biofolate content of normal plasma from 4 subjects before and after exhaustive dialysis in Visking casing is represented in table I. The

Comparison between the Unsaturated Plasma Folate Binder and *in vivo* Labelled Plasma Folate Binder

F P RETIEF A DU P HEYNS, M OOSTHUIZEN O R VAN REENEN and R. OELOFSE

Departments of Internal Medicine, Haematology and Biophysics,
Faculty of Medicine, University of the Orange Free State, Bloemfontein

Key Words. Plasma folate binders Acid medium Dialysis Chromatography

Abstract Endogenous plasma folate binder denuded of folate by dialysis at pH 3, subsequently bound more methylfolate than folic acid, in contrast with the minor unsaturated plasma binder which bound folic acid in preference to methylfolate. On Sephadex DEAE A50 chromatography $^{14}\text{C-CH}_3\text{H}_4\text{PteGlu}$ bound to acid-denuded endogenous binder eluted like the endogenous binder radioactivity labelled *in vivo* after oral $^{14}\text{C-CH}_3\text{H}_4\text{PteGlu}$. It is suggested that the endogenous plasma folate binder is not identical with the unsaturated binder.

It is still uncertain whether the binder of endogenous plasma folate [1 2] and the minor unsaturated folate binder demonstrable by *in vitro* addition of folate to plasma [3-7] represent the same substance. *In vivo* labelling of the endogenous binder [8] showed N5 methyltetrahydrofolate ($5\text{CH}_3\text{H}_4\text{PteGlu}$) to bind in preference to folic acid (PteGlu) whereas *in vitro* work showed oxydised folates to bind more avidly to the unsaturated binder than did reduced (physiological) folates [4 5]. However KAMEN and CASTON [9] making use of acid dissociation of the folate-binder complex [10] reported that the endogenous binder of neonatal serum also binds PteGlu in preference to $5\text{CH}_3\text{H}_4\text{PteGlu}$. Their acidification procedure was associated with considerable unexplained loss of binding capacity.

In the present study features of the unsaturated binder are further compared with those of the acid-dissociated and *in vivo* labelled endogenous binder.

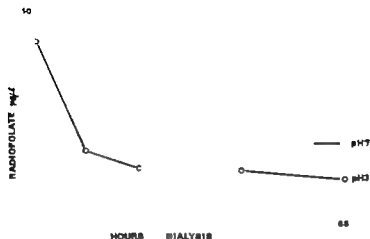


Fig. 1 Results of prolonged Visking dialysis at pH 3 on *in vivo* bound radiofolate in plasma of subject 2. Result of exhaustive dialysis at pH 7 on the same plasma is also given.

Table II. Effect of acidification dialysis on *in vivo* bound radiofolate after isotope ingestion

Subjects (sample, hours postabsorption)	Radiofolate, $\mu\text{g/l}$		After dialysis at pH 3 $^{14}\text{C } 5\text{CH}_3\text{H}_4\text{PteGlu}$
	Initial $^{14}\text{C } 5\text{CH}_3\text{H}_4\text{PteGlu}$		
	Total	Bound	
Subject 1 (6 h)	9.6	4.1	2.6
Subject 2 (6 h)	8.6	3.5	1.6
Subject 3 (24 h)	1.4	1.1	0.04

Dialysis-resistant at pH 7

saturated folate binding capacity). When this plasma was chromatographed the bound fraction, containing $^{14}\text{C } 5\text{CH}_3\text{H}_4\text{PteGlu}$ only eluted like the *in vivo* bound radiofolate. There was a minor $^3\text{H-PteGlu}$ peak which appeared in the elution position of free PteGlu. No definite protein binding of PteGlu could thus be demonstrated in this system.

Table I *In vitro* binding of radiofolates to normal plasma

Normal subjects	Biofolate, $\mu\text{g/l}^1$		Radiofolate bound, $\mu\text{g/l}$		dialysis pH 3/7 ²				
	total	bound	dialysis pH 7 ²		total	dialysis pH 3/7 ²			
			³ H PteGlu	¹⁴ C-CH ₃ H ₄ PteGlu		³ H PteGlu	¹⁴ C-CH ₃ H ₄ PteGlu	to ³	
1	5	3	0.09	0.01	0.10	0.2	0.76	0.	
2	9	3	0.12	0.03	0.15	0.47	1.00	1.4	
3	45	3	0.12	0.04	0.16	0.68	1.73	2.4	
4	6	2	0.13	0.06	0.19	0.36	2.40	2.7	

¹ Plasma biofolate before and after exhaustive Visking dialysis.² Radiofolates added at pH 7³ Radiofolates added at pH 7 after previous plasma dialysis at pH 3

nondialysable fraction represents the endogenous binder. The unsaturated radiofolate-binding capacity for folic acid and methylfolate at pH 7 is also recorded. These plasmas were then dialysed at pH 3 for 24 h, the pH neutralized by dialysis against buffer with pH 7, and the unsaturated radiofolate-binding capacity determined at this pH. After acidification and exhaustive dialysis to remove folate liberated from the folate/endogenous binder complex at low pH, methylfolate binding increased considerably more (0.01–0.06 to 0.76–2.40 $\mu\text{g/l}$) than did folic acid binding (0.09–0.13 to 0.22–0.68 $\mu\text{g/l}$).

In vivo Binding

The rate of radiofolate clearance during prolonged dialysis at pH 3 (plasma of subject 2) is demonstrated in figure 1, showing that loss of *in vivo* bound radiofolate is near maximal at 12 h, but that a substantial fraction is dialysis-resistant. The effect of 24 h dialysis at pH 3 on *in vivo* bound plasma radiofolate in 3 subjects is depicted in table II.

Chromatography

The plasma of subject 1, taken 6 h after ¹⁴C-5CH₃H₄PteGlu ingestion, was chromatographed before and after exhaustive dialysis at pH 7. The bound (dialysis-resistant) radiofolate fraction eluted in the position of albumin (fig. 2). Radioactive folic acid and methylfolate were added to normal plasma (predialysed for 24 h at pH 3), incubated for 30 min at 37 °C, and then dialysed for 24 h at pH 7 (as for the determination of un

bind folate [5] could not be confirmed [23]. Evidence exists that this binder may withhold folate from living cells [5-18], but its true functional significance remains obscure. It is present in normal plasma or serum in minor quantities only rarely rising above $1 \mu\text{g/l}$ [5-7, 12]. It has been suggested that the unsaturated binder is structurally similar to the saturated binder of endogenous folate [9, 15, 16, 24].

The binder of endogenous plasma folate has been reported to bind approximately one-third [19, 20] to one-half [15] of plasma folate, or $1-5 \mu\text{g/l}$ in normal plasma [2]. The main endogenous binder is chromatographically albumin-associated [8, 13, 19, 20] on DEAE A50 columns, although minor binders associated with α_2 -macroglobulins [21] and even transferrin [22] have been suggested. After dissociating folate at acid pH from cord serum binder JAMES and CASTON [9] found a denatured protein with molecular mass of 40,000, a binding preference for PteGlu rather than $\text{CH}_2\text{H}_2\text{PteGlu}$, but a binding capacity only 10% of the original serum binder. Also using acid-dissociation to study the normally saturated plasma binder COLMAN and HERNERT [24] similarly found low total folate binding capacities of less than $0.5 \mu\text{g/l}$. They utilized only PteGlu as test folate and found values of the same order as was recorded with PteGlu in our limited study (table I). With $\text{CH}_2\text{H}_2\text{PteGlu}$ our binding capacities were considerably higher and one might suspect that COLMAN and HERNERT [24] could have registered similar findings had this folate been used instead of PteGlu.

Table I shows that total plasma radiofolate binding after acidification dialysis (consisting predominantly of $\text{CH}_2\text{H}_2\text{PteGlu}$ binding), approached the original total biofolate binder in 3 of the 4 normal plasmas tested. That the method used does not cause complete folate-binder dissociation, is confirmed by table II and figure 1 where the *in vivo* bound radiofolate could not be totally cleared by dialysis at low pH. Our better radiofolate binding even after incomplete dissociation of the folate-binder complex, as compared with other workers [9, 24] is possibly based on better preservation of binder protein, inherent in the more gradual dialysis acidification process. Prolonged acidification which may damage the milk folate binder [25] obviously did not destroy the plasma binder. Another explanation for better binding with the present method may be that Visking dialysis is less disruptive on the *in vitro* folate-binder complex than charcoal adsorption [9]. We have previously shown that charcoal adsorption and exhaustive dialysis do not give comparable results in the study of plasma folate binding [13]. Less efficient dialysis clearance of free radiofolate would not

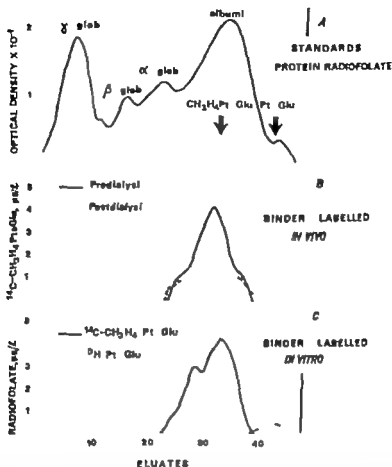


Fig 2 Results of DEAE-A50 liquid chromatography are represented a Typical plasma protein elution pattern with indication of elution of PtGlu and $\text{CH}_3\text{H}_4\text{PtGlu}$ standards. b *In vitro* labelled plasma radiofolate (subject 1) with dialysis-resistant (bound) radiofolate fraction. c Radiofolate bound *in vitro* to plasma after previous acid-dissociation of endogenous folate from folate-binder complex.

Discussion

The unsaturated plasma folate binder has a molecular mass less than 100 000 [5] and is probably albumin [14–15] although a macromolecular binder with molecular mass in excess of 200 000 has been described in folate deficiency [16]. Oxidized folates are bound in preference to reduced (physiological) folates [5–17] and binding of folic acid is pH dependent and maximal at pH 6–7.4 [14–16]. Claims that transferrin may

bind folate [5] could not be confirmed [23]. Evidence exists that this binder may withhold folate from living cells [5-18] but its true functional significance remains obscure. It is present in normal plasma or serum in minor quantities only rarely rising above $1 \mu\text{g/l}$ [5-7, 12]. It has been suggested that the unsaturated binder is structurally similar to the saturated binder of endogenous folate [9, 15, 16, 24].

The binder of endogenous plasma folate has been reported to bind approximately one-third [19, 20] to one-half [15] of plasma folate, or $1-5 \mu\text{g/l}$ in normal plasma [2]. The main endogenous binder is chromatographically albumin-associated [8, 13, 19, 20] on DEAE-A50 columns, although minor binders associated with α_2 -macroglobulins [21] and even transferrin [22] have been suggested. After dissociating folate at acid pH from cord serum binder KAMEN and CASTON [9] found a denatured protein with molecular mass of 40,000, a binding preference for PteGlu rather than $\text{CH}_3\text{H}_2\text{PteGlu}$, but a binding capacity only 10% of the original serum binder. Also using acid-dissociation to study the normally saturated plasma binder COLMAN and HERBERT [24] similarly found low total folate binding capacities of less than $0.5 \mu\text{g/l}$. They utilized only PteGlu as test folate and found values of the same order as was recorded with PteGlu in our limited study (table I). With $\text{CH}_3\text{H}_2\text{PteGlu}$ our binding capacities were considerably higher and one might suspect that COLMAN and HERBERT [24] could have registered similar findings had this folate been used instead of PteGlu.

Table I shows that total plasma radiofolate binding after acidification dialysis (consisting predominantly of $\text{CH}_3\text{H}_2\text{PteGlu}$ binding), approached the original total biofolate binder in 3 of the 4 normal plasmas tested. That the method used does not cause complete folate-binder dissociation, is confirmed by table II and figure 1 where the *in vivo* bound radiofolate could not be totally cleared by dialysis at low pH. Our better radiofolate binding even after incomplete dissociation of the folate-binder complex, as compared with other workers [9, 24] is possibly based on better preservation of binder protein, inherent in the more gradual dialysis acidification process. Prolonged acidification which may damage the milk folate binder [25] obviously did not destroy the plasma binder. Another explanation for better binding with the present method may be that Visking dialysis is less disruptive on the *in vivo* folate-binder complex than charcoal adsorption [9]. We have previously shown that charcoal adsorption and exhaustive dialysis do not give comparable results in the study of plasma folate binding [13]. Less efficient dialysis clearance of free radiofolate would not

underly the greater radiofolate retention as this would not explain the preferential binding of the physiological methyl-folate over folic acid.

In view of the fact that the unsaturated plasma binder preferentially binds PteGlu, while the endogenous (normally saturated) binder has a greater affinity for reduced physiological folates, both *in vitro* (table I) and *in vivo* [8-13] this limited study suggests that these two binders are probably not identical. With the relatively small DEAE columns used in this study we were unable to demonstrate PteGlu protein binding either before or after acid dissociation of the endogenous folate-binder complex. The minor ^3H peak visible on the lower graph in figure 2 probably represents residual ^3H PteGlu not cleared by dialysis, rather than binding to prealbumin.

Acknowledgements

This project was assisted by an ad hoc research grant from the SA Medical Research Council.

References

1. MARKKANEN T. Pteroylglutamic acid (PGA) activity of serum in gel filtration. *Life Sci.* 7: 887-895 (1968).
2. RETEIF F. F. and HENDERSON, Y. J.. Folate binders in body fluids. *J. clin. Path.* 23: 703-707 (1970).
3. METZ, J. ZALUSKY R., and HERBERT V.. Folic acid binding by serum and milk. *Am. J. clin. Nutr.* 21: 289-297 (1968).
4. ROTHENBERG, S. P. and DA COSTA, M. Further observations on the folate-binding factor in some leukemic cells. *J. clin. Invest.* 50: 719-726 (1971).
5. WAXMAN, S. and SCHREIBER, C. Characteristics of folic acid-binding protein in folate-deficient serum. *Blood* 42: 291-301 (1973).
6. ZETTLER, A. and DULY P. H. New evidence for a binding principle specific for folates as a normal constituent of human serum. *Clin. Chem.* 20: 1313-1319 (1974).
7. EXCHNER, E. R. PAINE, C. J. DICKSON V. L., and HARGROVE, M. D. Chemical and laboratory observations on serum folate-binding protein. *Blood* 46: 599-609 (1975).
8. RETEIF F. F. HEYNS, A. du P. OOSTHUIZEN M., and VAN REINEN, O. R.. *In vivo* plasma and urine folate binding after ingestion of ^3H folic acid and ^1C -methylfolate. *Br. J. Haemat.* 33: 415-424 (1976).
9. KAMEN, B. A. and CASTON J. D. Purification of folate binding factor in normal umbilical cord serum. *Proc. natn. Acad. Sci. USA* 72: 4261-4264 (1975).

- 10 COLMAN, N. and HERRERT V. Measurement of total folate binding capacity (TFBC) of serum after removal of endogenous folate at acid pH. *Chn. Res.* 22, 700A (1974).
- 11 HERRERT, V.: Aseptic addition method for L. casei assay of folate activity in human serum. *J. clin. Path.* 19, 12-16 (1966).
- 12 RETTEF, F. F., HEYNS, A. du P., OOSTHUIZEN, M., VAN REEDEN, O. R., and RADEN-MOEST, C. J. *In vitro* binding of folates by body fluids. *Br. J. Haemat.* 32, 113-121 (1976).
- 13 RETTEF, F. P.; HEYNS, A. du P., OOSTHUIZEN, M., and VAN REEDEN, O. R.. Aspects of folate metabolism in renal failure. *Br. J. Haemat.* 36, 405-415 (1977).
- 14 ELKORN, L. Binding of folic acid by human plasma proteins. *Acta haemat.* 48, 207-212 (1972).
- 15 SOLIMAK, H. A. and OLESEN, H.. Folic acid binding by human plasma albumin. *Scand. J. clin. Lab. Invest.* 36, 299-304 (1976).
- 16 WAXMAN, S.: Folate binding proteins. *Br. J. Haemat.* 29, 32-39 (1975).
- 17 DA COSTA, M. and ROTTERDAM, S. P. Appearance of a folate binder in leukocytes and serum of women who are pregnant or taking oral contraceptives. *J. Lab. clin. Med.* 83, 207-214 (1974).
- 18 THOMAS, G. and HERRERT V. Inhibition by human serum, Dilantin, and methotrexate (MTX) and enhancement by 2-deoxy-glucose of ^3H -pteroylglutamic acid (^3H PGA) and ^3H 5-methyltetrahydrofolic acid (M-THF) uptake by human bone marrow cells *in vitro*. *Chn. Res.* 19, 433 (1977).
- 19 MARKKANEN, T. and PELTOLA, O.: Binding of folic acid activity by body fluids. *Acta haemat.* 43, 272-279 (1970).
- 20 MARKKANEN, T. and PELTOLA, O.: Carrier proteins of folic acid activity in human serum. *Acta haemat.* 45, 106-111 (1971).
- 21 MARKKANEN, T., PAJULA, R. L., VIRTANEN, S., and HIMANEN, P.: New carrier protein(s) of folic acid in human serum. *Acta haemat.* 48, 145-150 (1972).
- 22 MARKKANEN, T.; VIRTANEN, S., HIMANEN, P. and PAJULA, R.-L.. Transferrin, the third carrier protein of folic acid activity in human serum. *Acta haemat.* 48, 213-217 (1972).
- 23 JACOB, E. and HERRERT V. Evidence against transferrin as binder of either vitamin B₁₂ or folic acid. *Blood* 43, 767-768 (1974).
- 24 COLMAN, N. and HERRERT V. Total folate binding capacity of normal human plasma, and variations in uremia, cirrhosis and pregnancy. *Blood* 48, 911-921 (1976).
- 25 FORD, J. E.; SALTER, D. N., and SCOTT, K.. The folate binding protein in milk. *J. Dairy Res.* 36, 435-447 (1969).

Study of Amino Groups of the Human Platelet Membrane¹

J F STOLTZ and A. NICOLAS

Groupe de recherche d'hémorhéologie, Centre régional de transfusion et
d'hématologie, Brubois, Vandœuvre-lès-Nancy

Key Words. Platelet Amino groups Electrokinetic charge Cell electrophoresis

Abstract. The authors attempted to evaluate different reagents in order to quantify the amino groups of the platelet membrane.

The study was carried out by the action of different aldehydes and acid anhydrides. The use of 2,4,6-trinitrobenzenesulfonic acid made possible an approach by electrophoresis and chemical assay

The results showed an increase in the surface charge corresponding to a mean number of amino groups varying from $1.86 \cdot 10^6$ groups with citraconic acid to $3.94 \cdot 10^6$ groups with acetaldehyde.

By analogy with researches achieved in colloidal systems, the ionized or ionizable groups on the cell membrane are thought to play a significant part in cell contact. The nature of anionic sites on cell membrane has been extensively studied but comparatively few works have been published on cationic sites [6]

In physiological conditions, human blood platelets are negatively charged [5 7 11] and many works have pointed out its importance in the physicochemical stability of cellular suspensions [15] The study of the action of neuraminidase and acetaldehyde upon blood platelets has led to a preliminary repartition between anionic and cationic groups.

¹ This work was supported by DRME - Biological Section, Grant No 76.34 146 00.480.75 01

In this communication we have attempted to evaluate different methods to approach the amino groups of the platelet surface [17]

Material and Methods

Preparation of Platelet Suspensions

Platelet suspensions were prepared from whole blood of healthy donors, from the Regional Blood Transfusion Center. The sample was drawn in siliconized glass and slowly centrifuged at 18°C. The platelet-rich plasma (PRP) thus obtained, depleted of erythrocytes, was separated and centrifuged at 3,000 g for 15 min.

The platelet pellet was washed twice in buffered saline (pH = 7 by use of NaHCO₃) and resuspended in order to obtain final concentration of 2.3×10^9 platelets/mm³ for the chemical estimations, and $2-3 \times 10^6$ /mm³ for the electrophoretic studies.

Electrophoretic Study

The electrophoretic mobility was determined using a liquid phase electrophoresis apparatus with a rectangular cell [14]. The surface charge was calculated from Gouy-Chapman theory while the membrane potential taken equal to the zeta potential, was determined by Helmholtz Smolachowski's formula. In all determinations of surface charge, we have kept constant ionic strength. For the calculations, platelet surface is taken equal to $28.3 \cdot 10^{-3}$ cm².

Reagents

The study of amino groups was generally carried out using electrophoresis after action of different aldehydes and acid anhydrides [9]. In addition, we used 2,4,6-trinitrobenzenesulfonic acid (TNBS) which makes possible an approach by electrophoresis and chemical assay.

Aldehydes. We chose to study the action of acetaldehyde (Merck), since it has been employed for a long time, and that of glutaraldehyde (Serva). This latter is a dialdehyde which reacts rapidly with amino groups [2, 4] and, in addition, it has little effect on cellular structures [12, 13].

Acid anhydrides. We studied the action of acetic and citraconic anhydrides (Merck, Fluka). The latter is a cyclic anhydride well known for its specificity towards amino groups.

For these two classes of reagents, the pH of platelet suspensions was adjusted to 7.35 by use of Sorremsen phosphate buffer [12] and the incubation time was 1 h at room temperature except for acetaldehyde (4°C).

2,4,6-Trinitrobenzenesulfonic Acid. The action of TNBS (Sigma) with amino groups is relatively complex [3] but very specific.

The N-trinitrophenyl derivatives being able to form complexes with the sulfhydryl groups released during the reaction, we ought to wash the cells and resuspend them in physiological saline before their electrophoretic mobility was measured.

For spectrophotometric assay the Fields protocol is used [3] and absorption is measured at the wavelength of 420 nm.

Study of Amino Groups of the Human Platelet Membrane¹

J. F. STOLTZ and A. NICOLAS

Groupe de recherche d'hémorhéologie, Centre régional de transfusion et
d'hématologie, Brabois, Vandœuvre lès-Nancy

Key Words. Platelet · Amino groups · Electrokinetic charge · Cell electrophoresis

Abstract The authors attempted to evaluate different reagents in order to quantify the amino groups of the platelet membrane.

The study was carried out by the action of different aldehydes and acid anhydrides. The use of 2,4,6-trinitrobenzenesulfonic acid made possible an approach by electrophoresis and chemical assay.

The results showed an increase in the surface charge corresponding to a mean number of amino groups varying from $1.86 \cdot 10^3$ groups with citraconic acid to $3.94 \cdot 10^6$ groups with acetaldehyde.

By analogy with researches achieved in colloidal systems, the ionized or ionizable groups on the cell membrane are thought to play a significant part in cell contact. The nature of anionic sites on cell membrane has been extensively studied but comparatively few works have been published on cationic sites [6].

In physiological conditions, human blood platelets are negatively charged [5, 7, 11] and many works have pointed out its importance in the physicochemical stability of cellular suspensions [15]. The study of the action of neuraminidase and acetaldehyde upon blood platelets has led to a preliminary repartition between anionic and cationic groups.

This work was supported by DRME - Biological Section, Grant No. 76.34.146.00.480.75.01.

Received, October 10, 1977; accepted, April 12, 1978.

We were able to show that the ratio between total amino groups obtained by assay of platelets previously treated with SDS and superficial groups varies between 2.5 and 3.5. It may thus be estimated that the superficial groups represent 30–40% of total platelet amino groups.

Discussion and Conclusion

The reaction of acetaldehyde with protein amino groups may be explained by the formation of a Schiff's base. However, it is relatively slow and even with a large excess of reagent, we do not obtain total reactivity of all the amino groups. In a similar study the figures found by *Sesman* [10] pointed out an increase of the platelet surface charge (20%) and a number of groups equal to $3.5 \times 10^5/\text{cell}$.

According to *Wold* [18] glutaraldehyde in aqueous solution forms a trimer which reacts simultaneously with two amino groups by bridging. This fact may explain our results. Indeed, the double reciprocal representation shows two straight lines which signify a biphasic action. In a first stage, the rate of the reaction is rapid as expected on former works [1, 2] and we note an increase in charge of 17% representing 80% of the total variation observed. In the second phase, the surface platelet being highly packed, the reaction is slower and the reached maximum of action corresponds to $3.43 \cdot 10^5$ amino groups/cell.

By reacting with amino groups, acetic anhydride induces the appearance of an amide function. On the other hand, the action of citraconic anhydride, which is a diacid anhydride, is associated with replacement of the positively charged amino group by an ionizable carboxyl group. Also, we should expect a variation of charge more important than with the use of the former reagents. However the results are quite similar. A different specificity between aldehydes and acid anhydrides is not a satisfactory explanation since anhydride acetic and acetyldehyde react in the same way. Thus, we must accept that the observed increase in charge is insufficient and several hypotheses may be stated. (a) ionization of the grafted carboxyl group is not complete at pH 7 used for the measurement of electrophoretic mobility. (b) a partial reversibility of the reaction: but working at pH 3–4 during incubation times varying from 1 to 3 h, we cannot obtain this reversibility. (c) partial solubilization of membrane proteins produced by an increase of the overall negative charge. This fact has been reported by different authors when maleic and succinic anhydrides were used [8, 16].

Results

Electrophoretic Study

Results concerning the different reagents showed in all cases, an increase of the electrophoretic mobility varying between 18.5 (acetaldehyde) and 22.8% (TNBS) (table I)

In order to obtain a more quantitative assessment of this action on the platelet superficial charge we express our results by using double reciprocal transformation. The representation of $\sigma_e/\Delta\sigma$ versus $1/(\text{reagent concentration})$ is linear and allows us to calculate the variation in charge at infinite concentration. The calculated figures are very close to that observed experimentally except in the case of acetaldehyde. This discrepancy is explained by a slower rate of reaction for this aldehyde confirmed by the important quantity of reagent required to observe a variation in charge equal to 10^6 (table I)

Spectrophotometric Assay

Amino groups were measured in 9 different platelet suspensions. For each one, we carried out 10 points of assay. The results obtained are linear in relation to platelet concentration. The number of amino groups was calculated taking as a reference the value of optical density corresponding to glycine controls.

The mean figures of the assays was $2.57 \pm 1.14 \times 10^3$ amino groups/cell and the extreme values 1.65 and 3.6×10^3 . This value represents the superficial groups of the membrane.

Table I. Determination of positively charged amino groups of the platelet membrane (comparison between five different molecules)

Molecules used	Doses used mM	Maxima variations of mobility %	Maxima variations of mobility (calculated) %	Mean number of groups per platelet	$\mu\text{mol of reagents}$ for $\frac{\Delta\sigma}{\sigma} = 0.1$
Acetaldehyde	1.70-26	18.5	23.9	3.94×10^6	4,200
Glutaraldehyde	0.0125-12.5	20.4	20.8	3.43×10^3	27
Acetic anhydride	0.005-2.5	21.7	14	3.53×10^3	57
Citraconic anhydride	0.11-4.4	21.2	22.5	1.86×10^3	30
TNBS	0.11-17	22.8	22.2	3.66×10^3	0.31

We were able to show that the ratio between total amino groups obtained by assay of platelets previously treated with SDS and superficial groups varies between 2.5 and 3.5. It may thus be estimated that the superficial groups represent 30–40% of total platelet amino groups.

Discussion and Conclusion

The reaction of acetaldehyde with protein amino groups may be explained by the formation of a Schiff's base. However, it is relatively slow and even with a large excess of reagent, we do not obtain total reactivity of all the amino groups. In a similar study, the figures found by Seaman [10] pointed out an increase of the platelet surface charge (70%) and a number of groups equal to $3.5 \times 10^3/\text{cell}$.

According to Wold [18] glutaraldehyde in aqueous solution forms a trimer which reacts simultaneously with two amino groups by bridging. This fact may explain our results. Indeed, the double reciprocal representation shows two straight lines which signify a biphasic action. In a first stage, the rate of the reaction is rapid as expected on former works [1, 2] and we note an increase in charge of 17% representing 80% of the total variation observed. In the second phase, the surface platelet being highly packed, the reaction is slower and the reached maximum of action corresponds to $3.43 \cdot 10^3$ amino groups/cell.

By reacting with amino groups, acetic anhydride induces the appearance of an amide function. On the other hand, the action of citraconic anhydride which is a diacid anhydride is associated with replacement of the positively charged amino group by an ionizable carboxyl group. Also we should expect a variation of charge more important than with the use of the former reagents. However, the results are quite similar. A different specificity between aldehydes and acid anhydrides is not a satisfactory explanation since anhydride acetic and acetyldehyde react in the same way. Thus, we must accept that the observed increase in charge is insufficient and several hypothesis may be stated: (a) ionization of the grafted carboxyl group is not complete at pH 7 used for the measurement of electrophoretic mobility; (b) a partial reversibility of the reaction, but working at pH 3–4 during incubation times varying from 1 to 3 h, we cannot obtain this reversibility; (c) partial solubilization of membrane proteins produced by an increase of the overall negative charge. This fact has been reported by different authors when maleic and succinic anhydrides were used [8, 16].

(d) the carboxyl groups are perhaps not all situated in the electrophoretic shear plane Z-E isometry due to the ethylenic double bond may explain that only 50% of the grafted groups are electrophoretically active (e) a last possibility would be the reaction of one molecule of citraconic anhydride with two amino groups. However the double reciprocal representation does not point out a biphasic action as in the case of glutaraldehyde which is known to react simultaneously with two amino groups.

Therefore if we consider the hypothesis of isometry to be the right one the number of groups detected by citraconic anhydride would be equal to $3.71 \times 10^5/\text{cell}$ that is quite close to the one obtained with other reagents.

Mehrishi [7] using citraconic and 2,3-dimethyl maleic anhydrides, has found variation in charge respectively equal to 27.5 and 25.3 μ .

The use of TNBS made it possible to obtain results both by electrophoretic and chemical techniques. The variation of the electrokinetic charge induced by TNBS is quite similar to that obtained with aldehydes and anhydrides. However TNBS is markedly more reactive. Chemical results show that TNBS is a relatively nonpenetrating reagent. The large higher number of groups obtained in these conditions may be explained by the fact that all the modified amino groups are not localized in the electrophoretic shear plane. This phenomenon is observed with all chemical groups when we compare these two different approaches [9].

References

- 1 BOWEN, J. H. and CATER, C. W. The interaction of aldehydes with collagen. *Biochim. Biophys. Acta* 168: 341-352 (1968).
- 2 CHIRITA, G. and CHIRALITA, D. Investigations of the reaction of aldehydes with the functional groups of some amino acids of the collagen structure. *J. Am. Leath. Chem. Ass.* 66: 369-384 (1971).
- 3 FIELDS, R. The measurement of amino groups in proteins and peptides. *Biochem. J.* 124: 581-590 (1971).
- 4 HANES, A. F. S. A. and HIRAMOTO, R. Reaction of proteins with glutaraldehyde. *Archs Biochem. Biophys.* 126: 16-26 (1968).
- 5 LARCAN, A., STOLTZ, J. F. et STREET, F. La charge électrique des éléments figurés du sang, p. 170 (Doin, Paris 1974).
- 6 MASON, R. G. and SHIFFRIN, R. W. Cell electrophoresis: reflections of platelet reactivity status. In *Platelet* chap. 8, pp. 1-3-131 (1971).
- 7 MEHRISHI, J. N. Molecular aspects of the mammalian cell surface. *Prog. biophys. mol. Biol.* 1: 1-67 (1972).

- 8 MOLDOW C. F. ZUCKER-FRANKLIN, D. GORDON, A., HOSPELBERG, V. and SILBER, R. Studies on the succinylation of erythrocyte membranes. *Biochim. biophys. Acta* 255, 133-148 (1972).
- 9 NICOLAS, A. Etude analytique des groupements chimiques membranaires ionisables des plaquettes sanguines humaines, thèse Nancy (1977).
- 10 SRAMAN, G. V. P. Surface potential and platelet aggregation: in *Platelets: their role in haemostasis and thrombosis*, pp. 53-68 (Schattauer Stuttgart 1967).
- 11 SRAMAN, G. V. P. and BROOK, D. E. Electrochemical aspects of platelet adhesion and aggregation. *Thromb. Diath. haemorrh.* 47 93-107 (1970).
- 12 SILVER, M. D. and GARDNER, H. A. Variation in rabbit platelet morphology induced by different aldehyde prefixatives. *Pathology* 2, 199-207 (1970).
- 13 SCOMA, J. J. LEISSNER, W. H., and GRUZE, H. J. Glutaraldehyde fixation of human blood platelets. *Thromb. Diath. haemorrh.* 27 272-277 (1972).
- 14 STOLTZ, J. F. STOLTZ, M., COLLIN, F. PETERS, A. et LARCAN, A. Méthode de mesure de la mobilité électrophorétique des particules colloïdales en suspension. Théorie et comparaison entre différentes cellules d'électrophorèse. *J. chem. Phys.* 66, 922-928 (1969).
- 15 STOLTZ, J. F. STOLTZ, M., PETERS, A., and LARCAN, A. Stability of the blood suspension and zeta potential of blood components; in *HARTERT and COPELEY Theoretical and clinical haemorrhéology* pp. 253-261 (Springer Berlin 1971).
- 16 VADLAMUDI, B. B. DZANDU, J. D. and LARWAY P. P. Differential solubilization of human erythrocyte cell membrane proteins by maleic anhydride. *Biochem. biophys. Res. Commun.* 64 64-71 (1975).
- 17 WEISS, L. and CUDNEY T. L. Some effects of pH and formaldehyde on the cellular electrokinetic surface. *Int. J. Cancer* 4 776-784 (1969).
- 18 WOLD, F. Bifunctional reagents. *Meth. Enzymol.*, vol. 23 pp. 623-651 (Academic Press, New York 1972).

(d) the carboxyl groups are perhaps not all situated in the electrophoretic shear plane Z-E isometry due to the ethylenic double bond may explain that only 50% of the grafted groups are electrophoretically active (e) a last possibility would be the reaction of one molecule of citraconic anhydride with two amino groups. However the double reciprocal representation does not point out a biphasic action as in the case of glutaraldehyde which is known to react simultaneously with two amino groups.

Therefore, if we consider the hypothesis of isometry to be the right one the number of groups detected by citraconic anhydride would be equal to $3.71 \times 10^5/\text{cell}$, that is quite close to the one obtained with other reagents.

Mehrishi [7] using citraconic and 2,3-dimethyl maleic anhydrides, has found variation in charge respectively equal to 27.5 and 25.3 μ .

The use of TNBS made it possible to obtain results both by electrophoretic and chemical techniques. The variation of the electrokinetic charge induced by TNBS is quite similar to that obtained with aldehydes and anhydrides. However TNBS is markedly more reactive. Chemical results show that TNBS is a relatively nonpenetrating reagent. The large higher number of groups obtained in these conditions may be explained by the fact that all the modified amino groups are not localized in the electrophoretic shear plane. This phenomenon is observed with all chemical groups when we compare these two different approaches [9].

References

- 1 BOWER, J. H. and CATER, C. W. The interaction of aldehydes with collagen. *Biochim. biophys. Acta* 168: 341-352 (1968).
- 2 CHIRITA, O. and CHIRALITA, II. Investigations of the reaction of aldehydes with the functional groups of some amino acids of the collagen structure. *J. Am. Leath. Chem. Ass.* 66: 369-384 (1971).
- 3 FIELDS, R. The measurement of amino groups in proteins and peptides. *Biochem. J.* 124: 581-590 (1971).
- 4 HARRIS, A. F. S. A. and HIRAMOTO, R. Reaction of proteins with glutaraldehyde. *Archs Biochem. Biophys.* 126: 16-26 (1968).
- 5 LARCAN, A., STOLTZ, J. F. et STREIFF, F. La charge électrique des éléments figurés du sang, p. 170 (Doin, Paris 1974).
- 6 MASON, R. D. and SHERMER, R. W. Cell electrophoresis: reflexions of platelet re-active status. In *Platelet*, chap. 8, pp. 123-131 (1971).
- 7 MEHRISHI, J. N. Molecular aspects of the mammalian cell surface. *Prog. biophys. mol. Biol.* 25: 1-67 (1972).

- 8 MOLDOW C. F. ZUCKER-FRANKLIN, D., GORDON, A., HORPELHORST, V. and SEITZ, R. Studies on the succinylation of erythrocyte membranes. *Biochim. biophys. Acta* 255 133-148 (1972).
- 9 NICOLAS, A. Etude analytique des groupements chimiques membranaires ionisables des plaquettes sanguines humaines; thèse Nancy (1977).
- 10 SEAMAN, G. V. P. Surface potential and platelet aggregation in Platelets: their role in haemostasis and thrombosis, pp. 53-68 (Schattauer Stuttgart 1967).
- 11 SEAMAN, G. V. P. and BROOK, D. E. Electrochemical aspects of platelet adhesion and aggregation. *Thromb. Diat. haemorrh.* 42. 93-107 (1970)
- 12 SILVER, M. D. and GARDNER, H. A. Variation in rabbit platelet morphology induced by different aldehyde prefixatives. *Pathology* 2. 199-207 (1970)
- 13 SOMMA, J. J. LIDWICK, W. H., and GRUZEK, H. J. Glutaraldehyde fixation of human blood platelets. *Thromb. diat. haemorrh.* 27 273-277 (1972).
- 14 STOLTZ, J. F. STOLTZ, M., COLLIN, P. PETERS, A. et LARCAN, A. Méthode de mesure de la mobilité électrophorétique des particules colloïdales en suspension. Théorie et comparaison entre différentes cellules d'électrophorèse. *J. chem. Phys.* 66 922-928 (1969).
- 15 STOLTZ, J. F. STOLTZ, M. PETERS, A., and LARCAN, A. Stability of the blood suspension and zeta potential of blood components; in HARTER and COMLEY Theoretical and clinical hemorheology pp. 253-261 (Springer Berlin 1971)
- 16 VADLAMUDI, B. P. DZANDU, J. D. and LARW Y P. F. Differential solubilization of human erythrocyte cell membrane proteins by maleic anhydride. *Biochem. biophys. Res Commun* 64 64-71 (1975).
- 17 WENIG, L. and CUDNEY T. L. Some effects of pH and formaldehyde on the cellular electrokinetic surface. *Int J Cancer* 4 776-784 (1969).
- 18 WOLD, T. Bifunctional reagents *Meth. Enzymol.* vol. 25, pp 623-651 (Academic Press, New York 1972).

Peripheral Platelet Count in Response to Salbutamol Before and After Adrenergic Beta Receptor Blockade

KRISTER FREDÉN LARS BERTIL OLSSON LARS VILÉN and JACK KUTTI

Department of Medicine Eastern Hospital, University of Gothenburg, Gothenburg

Key Words. Peripheral platelet count Splenic platelet pool ^{51}Cr Salbutamol
Metoprolol Propranolol

Abstract The effect of salbutamol (a comparatively selective adrenergic β receptor agonist) on the peripheral platelet concentration was studied before and after the ingestion of either 50 mg metoprolol or 40 mg propranolol. The study was carried out on healthy male volunteers and autologous ^{51}Cr -labelled platelets were employed at the experiments. Salbutamol was infused intravenously over a period of 6 min in a dose of $0.27 \text{ g kg}^{-1} \text{ min}^{-1}$. Prior to metoprolol and propranolol administration a statistically significant lowering in platelet bound radioactivity (PBR) was obtained in response to the salbutamol infusions. This salbutamol-induced fall in PBR was completely blocked by propranolol but was left unaffected by metoprolol. It is concluded that adrenergic β_2 -receptor stimulation induces a transient lowering of the peripheral platelet count.

Introduction

Intravenous infusions of isoprenaline (a non selective β receptor agonist) and salbutamol (a comparatively selective β_2 -receptor stimulator) have been shown to induce a significant lowering of the peripheral platelet concentration [10-11]. As regards salbutamol it was, however, observed that a decrease in the peripheral platelet concentration was not obtained unless a concomitant significant tachycardia was present [10]. Sal

butamol when given in high doses is known to cause a stimulation of the cardiac β_1 -receptors. Therefore it was suggested that the platelet lowering effect of salbutamol was mediated via β -receptors [10].

The aim of the present work was to characterize more precisely the adrenergic β -receptors involved in the above phenomenon. In the experiments healthy volunteers received intravenous infusions of salbutamol before and after the ingestion of either a selective β_1 receptor blocking agent (metoprolol) or a non-selective β -receptor antagonist (propranolol).

Material and Methods

10 healthy male volunteers were selected for the study. They had all given their informed consent, and the study was approved of by the local ethical committees.

5 subjects, aged 24–37 (mean 30) years, received intravenous infusions of salbutamol (Glaxo, Sweden) before and after an oral dose of 50 mg metoprolol tartrate (Seloken® Hänsle, Sweden). The remaining 5 subjects, aged 22–37 (mean 29) years, were given salbutamol intravenously before and after the ingestion of 40 mg propranolol hydrochloride (Inderal® ICI, England).

The day before the experiment autologous platelets were labelled *in vitro* with ^{51}Cr and were injected into the subjects. At the labelling procedure Ringer-citrate-dextrose solution [1] was used. The technique employed has been described elsewhere in detail [7]. On the following day (18 h) the subject arrived in the laboratory. Catheters were bilaterally inserted into the cubital veins, and the subject rested in supine position for 10 min. Thereafter two baseline blood samples were collected and the first infusion was started. The electrocardiogram was continuously monitored and the heart rate and blood pressure were recorded every 5 min.

Salbutamol in a dose of $0.27 \mu\text{g kg}^{-1} \text{ min}^{-1}$ diluted in 30 ml of saline was infused intravenously over a period of 6 min using constant pump flow device. 30 min after the end of the first infusion the subject received an oral dose of either 50 mg metoprolol or 40 mg propranolol. After 75 min, the second part of the experiment was started. After two baseline blood samples having been taken salbutamol was again infused in the same dose and during the same period of time before.

Venous blood was collected at 5 min intervals for the determination of platelet count, platelet-bound radioactivity (PBR) and haematocrit values. The enumeration of platelets was carried out by phase contrast microscopy [2]. The PBR was extracted from 9 ml of blood anticoagulated with 1 ml 3% EDTA as previously reported [7]. The haematocrit values were obtained by centrifugation of blood in capillary tubes at 13,460 g for 5 min (IEC MB Micro-Haematocrit Centrifuge).

Standard statistical methods were employed. Unless otherwise stated mean values \pm standard error of the mean (SEM) are reported. The statistical analysis was made using Student's *t* test and calculated on differences between matched pairs.

Peripheral Platelet Count in Response to Salbutamol Before and After Adrenergic Beta Receptor Blockade

KRISTER FREDÉN LARS BERTIL OLSSON LARS VILÉN and JACK KUTTI

Department of Medicine Eastern Hospital University of Gothenburg, Gothenburg

Key Words. Peripheral platelet count Splenic platelet pool ^{51}Cr Salbutamol Metoprolol Propranolol

Abstract The effect of salbutamol (a comparatively selective adrenergic β_2 -receptor agonist) on the peripheral platelet concentration was studied before and after the ingestion of either 50 mg metoprolol or 40 mg propranolol. The study was carried out on healthy male volunteers and autologous ^{51}Cr labelled platelets were employed at the experiments. Salbutamol was infused intravenously over a period of 6 min in a dose of $0.27 \text{ g kg}^{-1} \text{ min}^{-1}$. Prior to metoprolol and propranolol administration a statistically significant lowering in platelet-bound radioactivity (PBR) was obtained in response to the salbutamol infusions. This salbutamol induced fall in PBR was completely blocked by propranolol but was left unaffected by metoprolol. It is concluded that adrenergic β -receptor stimulation induces a transient lowering of the peripheral platelet count.

Introduction

Intravenous infusions of isoprenaline (a non-selective β receptor agonist) and salbutamol (a comparatively selective β_2 receptor stimulator) have been shown to induce a significant lowering of the peripheral platelet concentration [10-11]. As regards salbutamol it was, however, observed that a decrease in the peripheral platelet concentration was not obtained unless a concomitant significant tachycardia was present [10]. Sal

toprolol the infusion of salbutamol still caused a significant decrease in PBR ($p < 0.005$). Propranolol, on the other hand, completely abolished the effect of salbutamol on PBR.

No change in the haematocrit values occurred during the experiments.

Discussion

In a recent study we infused salbutamol in doses of 0.03 0.06 0.09 and $0.27 \mu\text{g kg}^{-1} \text{ min}^{-1}$ to a group of healthy subjects [10]. Those experiments showed that the 3 lower doses did not significantly affect the peripheral platelet count. However in response to $0.27 \mu\text{g kg}^{-1} \text{ min}^{-1}$ a statistically significant lowering of the peripheral platelet concentration occurred. Concomitantly there was an increase in the heart rate of about 25%. Isoprenaline, a pure β -receptor agonist with equal effects on β_1 and β_2 -receptors [3] induced a significant depression of the peripheral platelet count already when infused in a dose of $0.03 \mu\text{g kg}^{-1} \text{ min}^{-1}$ [11]. In those experiments a 35% increase in the heart rate was recorded.

Isoprenaline and salbutamol are known to be about equipotent *in vivo* with respect to the β_2 -receptor mediated effect on the human bronchial smooth muscles, but differ considerably with respect to the β -mediated effect on the heart [12, 13]. However salbutamol also possesses a weak β -receptor stimulating capacity. As stated above there was a considerable discrepancy between doses of isoprenaline and salbutamol needed to cause a change in the peripheral platelet count. It was therefore suggested that the increase in the heart rate and the lowering of the peripheral platelet count could be mediated via the same type of receptors, i.e. β receptors [10].

In the present work the effect of salbutamol was completely blocked by propranolol. After the administration of metoprolol, however there was still a significant ($p < 0.005$) depression of PBR. It is obvious that sufficient plasma concentrations of both blockers were reached as indicated by their effect on the basal heart rates. The doses of propranolol and metoprolol employed in the present study are also known to be equipotent with respect to exercise-induced tachycardia [6]. Therefore, it now seems evident that the salbutamol-induced lowering of PBR was mediated via β_2 and not β_1 receptors as previously suggested. In analogy with what is known from similar studies carried out with isoprenaline it seems highly

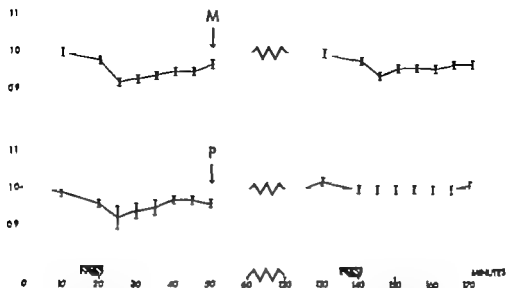


Fig 1 Platelet-bound radioactivity in response to intravenous infusions of salbutamol (hatched bars) given to healthy male volunteers before and after a oral dose of either 50 mg metoprolol (M) or 40 mg propranolol (P) (see text). The symbols denote the mean values \pm SE.

Results

Heart rate In response to the first infusion of salbutamol a 20% increase in the heart rate over basal values was recorded. Metoprolol caused a fall in basal heart rate from 68 ± 3 to 54 ± 1 bpm and propranolol from 70 ± 4 to 60 ± 3 bpm respectively. These differences between means were statistically significant ($p < 0.01$).

Platelet count The mean basal platelet count for the 10 healthy volunteers was 209 ± 37 (SD) range 139–254 $10^9/l$. There was no difference between the mean basal platelet counts in the two groups studied.

Platelet-bound radioactivity The results are shown in figure 1. The mean PBR in the first of the two baseline blood samples obtained prior to the first infusion of salbutamol were $3766 \pm 2,986$ (SD) and 1476 ± 534 (SD) cpm respectively. In figure 1 the PBR in the first baseline blood sample obtained before each infusion was arbitrarily set as 1.0 and all subsequent values were related to these baseline samples. In both groups the unblocked salbutamol infusions caused a significant ($p < 0.005$ and $p < 0.05$) decrease in PBR. After pretreatment with me-

toprolol the infusion of salbutamol still caused a significant decrease in PBR ($p < 0.005$). Propranolol on the other hand, completely abolished the effect of salbutamol on PBR.

No change in the haematocrit values occurred during the experiments.

Discussion

In a recent study we infused salbutamol in doses of 0.03, 0.06, 0.09 and $0.27 \mu\text{g kg}^{-1} \text{ min}^{-1}$ to a group of healthy subjects [10]. Those experiments showed that the 3 lower doses did not significantly affect the peripheral platelet count. However in response to $0.27 \mu\text{g kg}^{-1} \text{ min}^{-1}$ a statistically significant lowering of the peripheral platelet concentration occurred. Concomitantly there was an increase in the heart rate of about 25%. Isoprenaline, a pure β -receptor agonist with equal effects on β_1 and β_2 -receptors [3] induced a significant depression of the peripheral platelet count already when infused in a dose of $0.03 \mu\text{g kg}^{-1} \text{ min}^{-1}$ [11]. In those experiments a 35% increase in the heart rate was recorded.

Isoprenaline and salbutamol are known to be about equipotent *in vitro* with respect to the β_2 -receptor mediated effect on the human bronchial smooth muscles, but differ considerably with respect to the β -mediated effect on the heart [12, 13]. However salbutamol also possesses a weak β receptor stimulating capacity. As stated above there was a considerable discrepancy between doses of isoprenaline and salbutamol needed to cause a change in the peripheral platelet count. It was therefore suggested that the increase in the heart rate and the lowering of the peripheral platelet count could be mediated via the same type of receptors, i.e., β receptors [10].

In the present work the effect of salbutamol was completely blocked by propranolol. After the administration of metoprolol, however there was still a significant ($p < 0.005$) depression of PBR. It is obvious that sufficient plasma concentrations of both blockers were reached as indicated by their effect on the basal heart rates. The doses of propranolol and metoprolol employed in the present study are also known to be equipotent with respect to exercise-induced tachycardia [6]. Therefore it now seems evident that the salbutamol-induced lowering of PBR was mediated via β_1 and not β_2 -receptors as previously suggested. In analogy with what is known from similar studies carried out with isoprenaline it seems highly

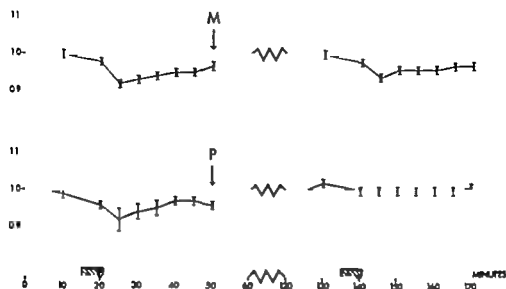


Fig 1 Platelet-bound radioactivity in response to intravenous infusions of salbutamol (hatched bars) given to healthy male volunteers before and after a oral dose of either 50 mg metoprolol (M) or 40 mg propranolol (P) (see text). The symbols denote the mean values \pm SE.

Results

Heart rate In response to the first infusion of salbutamol a 20% increase in the heart rate over basal values was recorded. Metoprolol caused a fall in basal heart rate from 68 ± 3 to 54 ± 1 bpm, and propranolol from 70 ± 4 to 60 ± 3 bpm respectively. These differences between means were statistically significant ($p < 0.01$).

Platelet count The mean basal platelet count for the 10 healthy volunteers was 209 ± 37 (SD) range 139–254 $10^9/l$. There was no difference between the mean basal platelet counts in the two groups studied.

Platelet bound radioactivity The results are shown in figure 1. The mean PBR in the first of the two baseline blood samples obtained prior to the first infusion of salbutamol were $3766 \pm 2,986$ (SD) and 1476 ± 534 (SD) cpm respectively. In figure 1 the PBR in the first baseline blood sample obtained before each infusion was arbitrarily set as 1.0 and all subsequent values were related to these baseline samples. In both groups the unblocked salbutamol infusions caused a significant ($p < 0.005$ and $p < 0.05$) decrease in PBR. After pretreatment with me

toprolol the infusion of salbutamol still caused a significant decrease in PBR ($p < 0.005$). Propranolol, on the other hand, completely abolished the effect of salbutamol on PBR.

No change in the haematocrit values occurred during the experiments.

Discussion

In a recent study we infused salbutamol in doses of 0.03, 0.06, 0.09 and $0.27 \mu\text{g kg}^{-1} \text{ min}^{-1}$ to a group of healthy subjects [10]. Those experiments showed that the 3 lower doses did not significantly affect the peripheral platelet count. However in response to $0.27 \mu\text{g kg}^{-1} \text{ min}^{-1}$ a statistically significant lowering of the peripheral platelet concentration occurred. Concomitantly there was an increase in the heart rate of about 25%. Isoprenaline, a pure β -receptor agonist with equal effects on β_1 and β_2 -receptors [3] induced a significant depression of the peripheral platelet count already when infused in a dose of $0.03 \mu\text{g kg}^{-1} \text{ min}^{-1}$ [11]. In those experiments a 35% increase in the heart rate was recorded.

Isoprenaline and salbutamol are known to be about equipotent *in vivo* with respect to the β receptor mediated effect on the human bronchial smooth muscles, but differ considerably with respect to the β -mediated effect on the heart [12, 13]. However salbutamol also possesses a weak β receptor stimulating capacity. As stated above there was a considerable discrepancy between doses of isoprenaline and salbutamol needed to cause a change in the peripheral platelet count. It was therefore suggested that the increase in the heart rate and the lowering of the peripheral platelet count could be mediated via the same type of receptors, i.e., β receptors [10].

In the present work the effect of salbutamol was completely blocked by propranolol. After the administration of metoprolol, however there was still a significant ($p < 0.005$) depression of PBR. It is obvious that sufficient plasma concentrations of both blockers were reached as indicated by their effect on the basal heart rates. The doses of propranolol and metoprolol employed in the present study are also known to be equipotent with respect to exercise-induced tachycardia [6]. Therefore, it now seems evident that the salbutamol induced lowering of PBR was mediated via β_2 and not β_1 receptors as previously suggested. In analogy with what is known from similar studies carried out with isoprenaline it seems highly

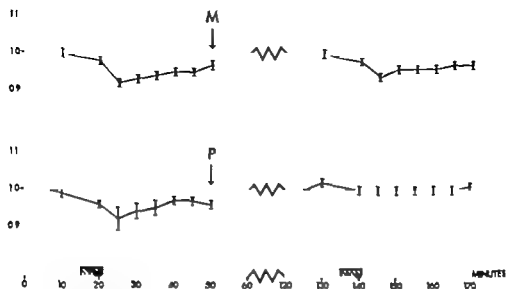


Fig 1 Platelet-bound radioactivity in response to intravenous infusions of salbutamol (hatched bars) given to healthy male volunteers before and after a oral dose of either 50 mg metoprolol (M) or 40 mg propranolol (P) (see text). The symbols denote the mean values \pm SE.

Results

Heart rate In response to the first infusion of salbutamol a 20% increase in the heart rate over basal values was recorded. Metoprolol caused a fall in basal heart rate from 68 ± 3 to 54 ± 1 bpm and propranolol from 70 ± 4 to 60 ± 3 bpm respectively. These differences between means were statistically significant ($p < 0.01$).

Platelet count The mean basal platelet count for the 10 healthy volunteers was 209 ± 37 (SD) range 139–254 $10^9/l$. There was no difference between the mean basal platelet counts in the two groups studied.

Platelet-bound radioactivity The results are shown in figure 1. The mean PBR in the first of the two baseline blood samples obtained prior to the first infusion of salbutamol were 3.766 ± 2.986 (SD) and 1.476 ± 0.534 (SD) cpm respectively. In figure 1 the PBR in the first baseline blood sample obtained before each infusion was arbitrarily set as 1.0 and all subsequent values were related to these baseline samples. In both groups the unblocked salbutamol infusions caused a significant ($p < 0.005$ and $p < 0.05$) decrease in PBR. After pretreatment with me

- 9 KUTTI, J. FREDÉN, K. MILLERÖ, P. E., and LUNDQVIST, P. The exchangeable splenic platelet pool in response to selective adrenergic beta-1-receptor blockade. *Br J Haematol* 37 277-282 (1977).
- 10 KUTTI, J. OLSSON, L.-B. LUNDQVIST, P. and FREDÉN, K. The peripheral platelet count in response to intravenous infusion of salbutamol. *Acta. med. scand.* 207 515-517 (1977).
- 11 OLSSON, L.-B., KUTTI, J. LUNDQVIST, P. and FREDÉN, K. The peripheral platelet count in response to intravenous infusion of isoprenaline. *Scand. J Haematol.* 37 213-216 (1976).
- 12 SVEDMYR, N., and THINBERG, B. The effects of salbutamol and isoprenaline on beta-receptors in patients with chronic obstructive lung disease. *Post-grad. med. J suppl.* 47 (1971).
- 13 THINBERG, G. Studies on beta-adrenergic-stimulating and blocking drugs in asthmatic patients (Elanders Boktryckeri, Kungälv 1975).

probable that this decrease in the peripheral platelet concentration is due to a transient trapping of platelets by the spleen [5]

Since a considerably higher dose of salbutamol compared to isoprenaline is needed to achieve a lowering in the peripheral platelet count, it could be hypothesized that an agonism between β_1 and β_2 receptors is present. This assumption finds support in recent observations made by us [4, 8, 9]. In those studies it was shown that 50 mg metoprolol given by mouth to healthy volunteers induces a significant increase in the peripheral platelet concentration. The average peak value was reached 150 min after the ingestion of the drug and the elevation was maintained more than 4 h. In asplenic patients, however, no such increment of the peripheral platelet count occurred [9]. A similar increase in the platelet count is also present after the ingestion of 40 mg propranolol (unpublished observations).

Acknowledgements

This study was supported by Gothenburg University and the Medical Society of Gothenburg. The authors wish to thank Miss Marianne Dahlberg for skilful technical assistance.

References

1. ABRAHAMSEN, A. F.. A modification of the technique for ^{51}Cr -labelling of blood platelets giving increased circulating platelet radioactivity. *Scand. J. Haematol.* 5: 53-63 (1968).
2. BRECHER, G., SCHNEIDERMAN, M. and CRONKITE, E. P.. The reproducibility and constancy of the platelet count. *Am. J. clin. Path.* 23: 15-26 (1953).
3. BRITTAIN, R. T., JACK, D. and RITCHIE, A. C.. Recent beta-adrenoceptor stimulants. *Adv. Drug. Res.* 5: 197-253 (1970).
4. FREDÉN, K., KUTTI, J., LUNDBERG, P. and VILÉN, L.. The relation between baseline and 150 min platelet counts in response to selective beta-1 receptor blockade. An experimental study on healthy subjects. *Blut* 35: 373-375 (1977).
5. FREDÉN, K., OLSSON, L. B., SUURKULA, M. and KUTTI, J.. The exchangeable splenic platelet pool in response to intravenous infusion of isoprenaline. *Scand. J. Haematol.* (in press).
6. JOHANSSON, G., NYBERG, G. and SÖLVELL, L.. Influence of metoprolol and propranolol on haemodynamic effects induced by physical work and isoprenaline. *Acta pharmac. tox.* 36: suppl. 5 (1975).
7. KUTTI, J. and WEINFELD, A.. Platelet survival in man. *Scand. J. Haematol.* 8: 336-346 (1971).
8. KUTTI, J., BERGSTRÖM, A. L., and LUNDBERG, P.. Metoprolol and the peripheral platelet count. *Acta haemat.* 58: 89-93 (1977).

- 9 KUTTI, J. FREDÉN, K., MELBERG, P. E., and LUNDQVIST, P. The exchangeable splenic platelet pool in response to selective adrenergic beta-1-receptor blockade. *Br J Haematol* 37 277-282 (1977).
- 10 KUTTI, J. OLSSON, L. B., LUNDQVIST, P. and FREDÉN, K. The peripheral platelet count in response to intravenous infusion of salbutamol. *Acta med. scand.* 201 515-517 (1977).
- 11 OLSSON, L.-B. KUTTI, J. LUNDQVIST, P. and FREDÉN, K. The peripheral platelet count in response to intravenous infusion of isoprenaline. *Scand. J Haematol.* 77 213-216 (1976).
- 12 SVEDMYR, N. and THINDBOM, G. The effects of salbutamol and isoprenaline on beta-receptors in patients with chronic obstructive lung disease. *Post-grad. med. J suppl* 47 (1971).
- 13 THINDBOM, G. Studies on beta-adrenergic-stimulating and blocking drugs in asthmatic patients (Elanders Boktryckeri, Kungälv 1975).

Acta haemat. 60 316-320 (1978)

Chronic Granulocytic Leukemia, Neutrophilic Type, with Paraproteinemia (IgA Type K)

DANIEL A. VOROBIOF JOSEPH BENJAMIN H. KAPLAN and
ALEXANDER DVLANSKY

Departments of Internal Medicine C and of Hematology Soroka Medical Center
Faculty of Health Sciences,
Ben-Gurion University of the Negev Beer Sheva

Key Words Chronic granulocytic leukemia Paraproteinemia Bence Jones
protein Transcobalamins Alkylating agents

Abstract A patient with chronic granulocytic leukemia neutrophilic type was followed for 28 months. A paraproteinemia, IgA type κ , and Bence Jones proteinuria (K) appeared without prior chemotherapy with alkylating agents.

Leukemia in which paraproteinemia was found was reported previously. The recent improvements in the management of multiple myeloma have been associated with longer survival times of patients with this disease. This might be related to the increase in the number of patients who developed acute leukemia. Most of the acute leukemias described in such patients have been monocytic or myelomonocytic and therefore presumably not derived from the neoplastic plasma cells themselves [1-3 & 9-15]. We had the opportunity to follow a patient who suffered from chronic granulocytic leukemia neutrophilic type who had paraproteinemia (IgA type κ) without any prior chemotherapy.

Case Report

A 70-year-old white male was admitted to our hospital on July 1976. He complained of weakness, night sweats and undefined abdominal pain.

Received June 6, 1977 accepted March 21 1978.



Fig 1 Granulocytic hyperplasia as seen in bone marrow aspiration. May-Grünwald-Giemsa. $\times 1,000$

Fig 2 Plasma cells in marrow aspiration. May-Grünwald-Giemsa. $\times 1,000$.

Table 1 Quantitative serum immunoelectrophoresis

	Values, mg/ml				
	normal	September 1976	November 1976	February 1977	March 1977
IgG	1,200 (\pm 300)	700	720	590	448
IgA	280 (\pm 60)	600	3 400	1 600	1 400
IgM	88 (\pm \pm 2)	22	44	46	42

On the physical examination, temperature, pulse and blood pressure were normal. No palpable lymph nodes were found. However splenomegaly 8 cm below the left subcostal margin, was found, without palpable liver. Hemoglobin was 11.4 g/dl, red blood cells $3.87 \times 10^9/\mu\text{l}$, hematocrit 34 %, leukocytes 19,000/ μl , platelets 160,000/ μl and reticulocytes 1.2%. Differential count was segmented neutrophils 64 %, band forms 24 %, lymphocytes 6% and monocytes 6%. Toxic granulation and Pelger Huët anomaly were seen in peripheral blood. Electrophoresis of hemoglobin was normal. Blood sedimentation rate was 70 mm in the first hour. Blood urea and electrolytes were normal. However blood uric acid was persistently increased, 9 mg%. Unilateral trephine bone biopsy from the left iliac crest and marrow aspirations revealed granulocytic hyperplasia (fig. 1). Bone marrow karyotype was normal and no Ph chromosome was found. Leukocyte alkaline phosphatase index was high 370 (normal 15-70). X-rays of skull, gastrointestinal tract, skeletal and intravenous pyelography were normal. Liver and rectal biopsy were normal. Bone scan (^{99}Tc) was normal too. Serum B_{12} was 1,050 pg/ml (normal, 500-800 pg/ml). Serum transcobalamins: TC_I 9,074 pg/ml (normal 200-350 pg/ml) TC_{II} 994 pg/ml (normal 400-800 pg/ml) TC_{III} 2,362 pg/ml (normal 170-300 pg/ml).

In November 1976, proteinuria of 2 g/day was documented. Immunoelectrophoresis identified this protein as Bence Jones protein (λ -chain) which was secreted at 500 mg/day. Bone marrow aspiration revealed hypercellularity granulocytic hyperplasia and increased amount of plasma cells (15% fig. 2). Quantitative serum immunoelectrophoresis revealed high levels of IgA (type λ) as depicted in table 1.

Discussion

Rare cases of chronic granulocytic leukemia, neutrophilic type, were reported previously [3 4 11-13]. Our patient had splenomegaly granulocytic hyperplasia in the bone marrow and was negative for the Ph chromosome. Leukocyte alkaline phosphatase was elevated as reported previously [4 11-13].

Furthermore, transcobalamin examination was diagnostic for a myeloproliferative disorder consistent with chronic granulocytic leukemia. Neutrophilic granulocytes have the capacity to produce excessive amounts of TC_1 (similar to eosinophilic granulocytes) [10].

Patients reported previously with a combination of leukemia superimposed on myeloma were treated with alkylating agents for prolonged periods [5, 7, 14]. However, such a combination of spontaneous appearance of paraproteinemia (IgA type K) with Bence Jones proteinuria (subtype k) [8] without any prior chemotherapy, was, to our knowledge, not recorded previously. Amyloidosis was not found (liver and rectal biopsy negative) and no other additional causes such as macroglobulinemia, chronic lymphocytic leukemia, lymphoma or adult Fanconi anemia were found. It is conceivable that, due to the prolonged and also benign course of chronic granulocytic leukemia of neutrophilic type [3] which was untreated for a prolonged period, a new clone-producing paraprotein appeared.

Is it a coincidence that our patient had two diseases which occurred simultaneously and was it a chance association of two independent diseases? As the incidence of the neutrophilic type of chronic granulocytic leukemia is not assessed in Israel [personal communication of Prof. B. MODAN, Sheba Medical Center] no answer can be given to this question. How it is related to the primary disease can as yet not be explained.

References

- 1 ALLEN, E. L., METZ, E. N. and BALCERZAK, S. B. Acute myelomonocytic leukemia with macroglobulinemia, Bence Jones proteinuria and hypercalcemia. *Cancer* 32, 1:1 124 (1973).
- 2 COHEN, S. L., DOORNBOUTH, H. and WHITELAW, A. O. L. Leukemia on myeloma. *Br med J* iv 490 (1971).
- 3 GALT, F. and BAIRD, A. H. *Leukemia*. 3rd ed. pp 754-755 (Grune & Stratton, New York 1974).
- 4 JACOBSON, I. M. and CLARK, A. M. A case of neutrophilic leukemia. *Am. J. med. Sci.* 49 72 (1965).
- 5 KARCOWSKA, R. K. Alkylating agents as leukemogens in myeloma. *Cancer* 33 1103 (1974).
- 6 KYLE, R. A., PIERRE, R. V. and B. YED, E. H. Multiple myeloma and acute myelomonocytic leukemia. *New Engl J Med* 283 11 1 1125 (1970).
- 7 KYLE, R. A., PIERRE, R. V. and B. YED, E. D. Multiple myeloma and acute leukemia associated with alkylating agents. *Ann intern Med* 125 185-19* (1975).

- 8 PERRY M. L. and KYLE, R. A. The clinical significance of Bence Jones proteinuria. *Mayo Clin. Proc* 50 234-238 (1975)
- 9 QUIRT I. C. HART G. D. and SOOTS, M. Leukemia on myeloma. *Br med. J* 1 248 (1972)
- 10 RACHMILEWITZ, B. RACHMILEWITZ, M. and GROSS, J. A Vit B₁₂ binder with transcobalamin I characteristics synthesized and released by human granulocytes *in vitro* *Br J Haemat.* 26 557-567 (1974)
- 11 RUBIN H. Chronic neutrophilic leukemia. *Ann. Intern. Med* 65 93-100 (1966).
- 12 SHENDO, T. SAKAI, C., and SHIBATA, A. Neutrophilic leukemia and blastic crisis. *Ann Intern. Med* 87 66-67 (1977)
- 13 TANZER, J., HAREL, P. BOIRON M. and BERNARD, J. Cytochemical and cytogenetics findings in a case of chronic neutrophilic leukemia of mature cell type. *Lancet* 1 387 (1964).
- 14 VIOLANTI, E. C. and SAITA, G. Benzene and leukemia. *New Engl. J Med* 271 872-876 (1964)
- 15 WEBB, J. A. N., BATMAN C. J. T. and DAVIES, J. D. Leukemia on myeloma *Br med. J* 1v 231 (1971)

Binding of Protoporphyrin and Haemin to Human Spectrin

G. H. BEAVEN and W. B. GRATZER

National Institute for Medical Research, and
Medical Research Council Cell Biophysics Unit, King's College, London**Key Words.** Spectrin Haemin Protoporphyrin Haemoglobin Binding

Abstract Haemin and protoporphyrin IX, but not bilirubin, are extensively bound by human spectrin. The absorption spectrum of the bound haemin is indicative of coordination of the iron by nitrogenous ligands in the protein. The protoporphyrin IX generates difference spectra on binding, which change with ligand:protein ratio, showing the existence of at least two structurally distinct types of site. The binding of both ligands is complex, and may be cooperative. Binding isotherms, based on spectrophotometric titrations, are given. Haemin and protoporphyrin IX also bind strongly to erythrocyte ghosts. At some strengths near physiological we can find no evidence of binding of haemoglobin to spectrin, as judged by sedimentation velocity, and it appears that reported interactions of this nature represent only non-specific binding at low ionic strength.

Spectrin is a high-molecular weight protein, present as a layer on the cytoplasmic surface of the erythrocyte membrane [11-17]. There is evidence that it plays an essential part in maintaining and controlling the shape of the cell [4, 20]. Spectrin is evidently capable of entering into specific interactions with other cell constituents. Thus it appears to interact with actin, present in the membrane [4, 20] and with at least one other membrane protein, not yet identified [3], as well as self-associating to form a network under appropriate conditions *in vitro* [18, 23] and in the cell [27]. It has also been reported [6] that haemoglobin binds to spectrin, though the conditions used were far from physiological. As regards interactions with small molecules, spectrin is reported to bind membrane phospholipids [13, 24], and we have also found a rather weak interaction

- 8 PERRY M. L. and KYLE, R. A. The clinical significance of Bence Jones proteinuria. *Mayo Clin. Proc.* 50: 234-238 (1975).
- 9 QUIRT I. C., HART G. D. and SOOTS, M. Leukemia on myeloma. *Br. med. J.* 1, 248 (1972).
- 10 RACHMILEWITZ, B., RACHMILEWITZ, M. and GROSS, J.. A Vlt B₁₂ binder with transcobalamin I characteristics synthesized and released by human granulocytes *in vitro*. *Br. J. Haematol.* 26: 557-567 (1974).
- 11 RUBIN H. Chronic neutrophilic leukemia. *Ann. Intern. Med.* 63: 93-100 (1966).
- 12 SHINDO, T., SAKAI, C., and SHIBATA, A. Neutrophilic leukemia and blastic crisis. *Ann. Intern. Med.* 87: 66-67 (1977).
- 13 TANZER, J., HAREL, P., BORRON M. and BERNARD J.. Cytochemical and cytogenetic findings in a case of chronic neutrophilic leukemia of mature cell type. *Lancet* 1: 387 (1964).
- 14 VIOLANTI, E. C. and SAITA, G.: Benzene and leukemia. *New Engl. J. Med.* 271: 877-876 (1964).
- 15 WEBB, J. A. N., BATEMAN C. J. T. and DAVIES, J. D. Leukemia on myeloma. *Br. med. J.* 1, 231 (1971).

Binding of Protoporphyrin and Haemin to Human Spectrin

G. H. BEAVEN and W. H. GRATZER

National Institute for Medical Research, and
Medical Research Council Cell Biophysics Unit, King's College, London**Key Words:** Spectrin · Haemin · Protoporphyrin · Haemoglobin · Binding

Abstract. Haemin and protoporphyrin IX, but not bilirubin, are extensively bound by human spectrin. The absorption spectrum of the bound haemin is indicative of coordination of the iron by nitrogenous ligands in the protein. The protoporphyrin IX generates difference spectra on binding, which change with ligand:protein ratio, showing the existence of at least two structurally distinct types of site. The binding of both ligands is complex, and may be cooperative. Binding isotherms, based on spectrophotometric titrations, are given. Haemin and protoporphyrin IX also bind strongly to erythrocyte ghosts. At ionic strengths near physiological we can find no evidence of binding of haemoglobin to spectrin, as judged by sedimentation velocity, and it appears that reported interactions of this nature represent only non-specific binding at low ionic strength.

Spectrin is a high-molecular weight protein, present as a layer on the cytoplasmic surface of the erythrocyte membrane [11-17]. There is evidence that it plays an essential part in maintaining and controlling the shape of the cell [4, 20]. Spectrin is evidently capable of entering into specific interactions with other cell constituents. Thus it appears to interact with actin, present in the membrane [4, 20] and with at least one other membrane protein, not yet identified [3], as well as self-associating to form a network under appropriate conditions *in vitro* [18, 23] and in the cell [27]. It has also been reported [6] that haemoglobin binds to spectrin, though the conditions used were far from physiological. As regards interactions with small molecules, spectrin is reported to bind membrane phospholipids [13, 24] and we have also found a rather weak interaction

with calcium ions [unpubl. data]. We show here that the protein binds both haemin and protoporphyrin IX extensively under physiological solvent conditions. It does not, however, interact detectably with bilirubin. It further appears that the reported interaction with haemoglobin is a non-specific effect, operating only at low ionic strength, and is insignificant in physiological conditions.

Materials and Methods

Spectrin was prepared from fresh human cells according to procedures previously described [10, 16]. Haemin, bilirubin and protoporphyrin IX dimethyl ester were obtained from British Drug Houses and Sigma Chemical Co. The ester was hydrolysed with acid to yield the free porphyrin [8] and examined for purity by thin-layer chromatography [8]. Binding assays were performed in 0.1 M sodium chloride, 0.05 M Tris, pH 7.6. The porphyrin derivatives were dissolved in 0.01 M sodium hydroxide, and their concentrations determined spectrophotometrically after dilution of the alkaline solution with water in the case of haemin and bilirubin, and with 3 M hydrochloric acid in the case of protoporphyrin IX. In these conditions the molar absorptivities were taken as 5.8×10^4 (385 nm), 5.2×10^4 (440 nm) and 2.62×10^4 (408 nm) respectively. Protein concentrations were determined spectrophotometrically using a specific absorptivity $E_{1\%}^{1\text{cm}}$ of 10.8 [14].

For spectrophotometric titrations, aliquots of concentrated stock solutions of the ligands were added with micropipettes to the sample cell containing spectrin and to the reference cell, containing the buffer. Because of time effects, adsorption to the walls of the cell and progressive denaturation through mixing, definitive titration curves were obtained by using a separate protein sample for each point. Time effects were generally complete in about 15 min. Circular dichroism was measured in a Cary 61 instrument, and absorption difference spectra in Perkin Elmer Coleman 575 and Cary 118 spectrophotometers, using expanded absorbance scales where necessary. Interactions with haemoglobin were studied in the analytical ultracentrifuge (Spinco model E). Spectrin in the same buffer as above was introduced into one cell, and a spectrin-haemoglobin mixture into another fitted with a wedge window. The spectrin concentrations were identical in the two cells (ca. 2 mg/ml). These samples were compared in the same velocity run, at 60 000 rpm using schlieren optics.

Binding of ligands to ghosts was observed in the following way: ghosts, prepared according to Dodge *et al* [7] and washed until pale, were brought into suspension by swirling, and sampled with a 1 ml automatic pipette. Samples of ligand were added, and after mixing the suspensions were centrifuged for 30 min at 70,000 G. The supernatant was removed and the concentrations of free ligand measured spectrophotometrically as described above using as reference the supernatant from a ghost sample containing no added ligand. Ghost concentrations were determined by dry weight, aliquots of the preparation prior to addition of buffer being sampled into vials and dried to constant weight *in vacuo*.

Results and Discussion

Figure 1 shows the absorption spectrum generated when haemin binds to spectrin. With the spectrin present in large excess, only the strongest binding sites will be occupied and all or most of the ligand will be bound. The spectrum of the bound haemin is characterised by a Soret band at 415 nm. This is markedly longer than the wavelength of the Soret band reported for monomeric haemin, viz. 403 nm [5, 17] which is also the position associated with haemin bound to serum albumin [2, 15] and h-gandin [26]. It is supposed that in these complexes the iron atom is not coordinated to protein side chains, and the spectrum arises from the sequestration of monomeric haemin at the binding site. The red-shift observed in the spectrin complex relative to free monomeric haemin suggests that here protein groups (most probably nitrogenous side chains) are coordinated to the haem iron atom. The shape of the difference spectrum remains essentially unchanged as the haemin:protein ratio is increased, which suggests that all binding sites are similar in character. At high protein:haemin ratio the molar absorptivity difference approaches about 6.0×10^4 and we use this value for estimating the concentration of bound haemin. Figure 2 shows binding profiles thus obtained.

Analysis of these data has proved unexpectedly difficult. If one assumes that the haemin is in the same state free as bound (i.e. disregarding the monomer-dimer equilibrium of the free haemin) the binding data may be represented by means of a Scatchard plot [22] which would indicate the presence of two strong and a number of weaker binding sites. If on the other hand, one takes the coupled monomer-dimer equilibrium into account, with a dissociation constant of 2.5×10^{-6} M at pH 7.6, derived from the relation of BROWN *et al.* [5] and the dissociation constant measured at pH 7.0 by TIPPING *et al.* [25] an anomalous Scatchard plot results. We are unable to determine whether this arises from experimental error (in consequence for example of adsorption of free haemin at low concentration of cell walls) from an inadequacy in the description of the behaviour of the free haemin, or from cooperativity of binding. We have not attempted to analyse the binding isotherms further but figure 2 shows a series of calculated curves for selected numbers of sites and the intrinsic binding constant giving the best fit, viz. 5×10^4 M⁻¹. It appears from these curves that the data are reasonably consistent with the presence in each molecule of spectrin (reckoned as a dimer of molecular weight 500,000) [10, 14] of some 15–20 independent sites with similar affinities.

with calcium ions [unpubl. data]. We show here that the protein binds both haemin and protoporphyrin IX extensively under physiological solvent conditions. It does not, however, interact detectably with bilirubin. It further appears that the reported interaction with haemoglobin is a non-specific effect, operating only at low ionic strength and is insignificant in physiological conditions.

Materials and Methods

Spectrin was prepared from fresh human cells according to procedures previously described [10, 16]. Haemin, bilirubin and protoporphyrin IX dimethyl ester were obtained from British Drug Houses and Sigma Chemical Co. The ester was hydrolysed with acid to yield the free porphyrin [8], and examined for purity by thin-layer chromatography [8]. Binding assays were performed in 0.1 M sodium chloride, 0.05 M Tris, pH 7.6. The porphyrin derivatives were dissolved in 0.01 M sodium hydroxide, and their concentrations determined spectrophotometrically after dilution of the alkaline solution with water in the case of haemin and bilirubin, and with 3 M hydrochloric acid in the case of protoporphyrin IX. In these conditions the molar absorptivities were taken as 5.8×10^4 (385 nm), 5.2×10^4 (440 nm) and 2.62×10^4 (408 nm) respectively. Protein concentrations were determined spectrophotometrically using a specific absorptivity $E_{1\text{cm}}^{1\%}$ of 10.8 [14].

For spectrophotometric titrations, aliquots of concentrated stock solutions of the ligands were added with micropipettes to the sample cell containing spectrin and to the reference cell, containing the buffer. Because of time effects, adsorption to the walls of the cell and progressive denaturation through mixing, definitive titration curves were obtained by using a separate protein sample for each point. Time effects were generally complete in about 15 min. Circular dichroism was measured in a Cary 61 instrument, and absorption difference spectra in Perkin Elmer Coleman 575 and Cary 118 spectrophotometers, using expanded absorbance scales where necessary. Interactions with haemoglobin were studied in the analytical ultracentrifuge (Spinco model E). Spectrin in the same buffer as above was introduced into one cell and a spectrin-haemoglobin mixture into another fitted with a wedge window. The spectrin concentrations were identical in the two cells (ca. 2 mg/ml). These samples were compared in the same velocity run, at 60,000 rpm, using schlieren optics.

Binding of ligands to ghosts was observed in the following way: ghosts, prepared according to Dooze *et al.* [7] and washed until pale, were brought into suspension by swirling, and sampled with a 1-ml automatic pipette. Samples of ligand were added, and after mixing the suspensions were centrifuged for 30 min at 70,000 G. The supernatant was removed and the concentrations of free ligand measured spectrophotometrically as described above using as reference the supernatant from a ghost sample containing no added ligand. Ghost concentrations were determined by dry weight, aliquots of the preparation, prior to addition of buffer being sampled into vials and dried to constant weight *in vacuo*.

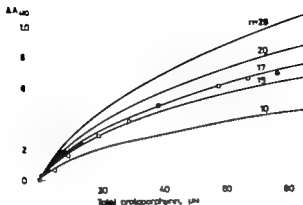


Fig 3 Spectrophotometric titrations of spectrin at 1.6 mg/ml with protoporphyrin IX. The points refer to experimental absorbance differences at 410 nm. The curves are calculated for the indicated numbers of identical sites per chain with an intrinsic binding constant of 10^4 M^{-1} for protoporphyrin IX monomer assuming molar difference absorptivity per ligand bound of 6.9×10^4 and protoporphyrin self-association constant of $2.5 \times 10^6 \text{ M}^{-1}$ (see text).

It should be stressed that this must represent a great oversimplification, since there is no reason to believe that spectrin should contain large numbers of identical binding sites or indeed, that the sites function independently. The data would not, however, justify the use of several floating parameters to generate a best fit. The binding isotherm is in fact sufficient to describe the binding properties in operational terms.

The binding of protoporphyrin IX was studied in a similar way. The absorption spectrum generated on binding is shown in figure 1. As might be expected, it resembles that of protoporphyrin-globin [21] and protoporphyrin albumin [15] at least in conditions of protein excess. The molar absorptivity difference on binding was estimated as 6.9×10^4 at 415 nm. In contrast to the spectrin-haemin system, however, the shape of the difference spectrum changes as more sites are occupied, indicating that there are at least two structurally distinct classes of site associated with absorption spectra with Soret bands at about 415 and 388 nm, respectively. Again, analysis is additionally complicated by a presumed dimerisation equilibrium of the free protoporphyrin IX, with a dissociation constant of $4 \times 10^{-4} \text{ M}$ [9]. Figure 3 shows calculated curves, based on binding of the monomeric species only and on a difference absorption spectrum corresponding only to the stronger type of site. At high ligand: protein ratio, the

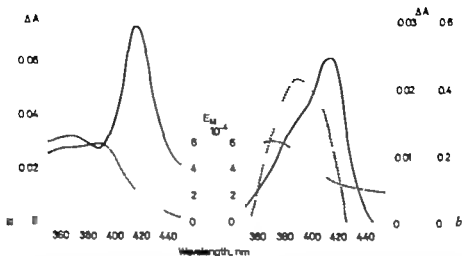


Fig 1 Spectral characteristics of haemin and protoporphyrin IX complexes of spectrin. *a* Spectrum of free haemin at pH 7.6 (---) (right-hand ordinate), and difference spectrum generated by addition of 2.25 M haemin to spectrin at 0.43 mg/ml (—) (left-hand ordinate). *b* Spectrum of free protoporphyrin IX at pH 7.6 (---) (left-hand ordinate) and difference spectra generated by addition of 1.5 M protoporphyrin (—) and of 37 μ M protoporphyrin (---) to spectrin at 0.50 mg/ml (right-hand ordinate scales, 0–0.03 and 0–0.6 respectively)

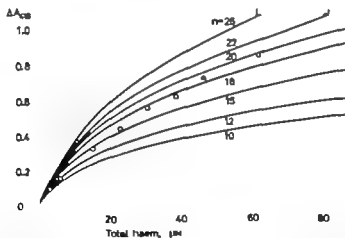


Fig 2 Spectrophotometric titrations of spectrin at 0.68 mg/ml with haemin. The points refer to experimental absorbance differences at 415 nm. The curves are calculated for the indicated numbers of identical sites per chain, with an intrinsic binding constant of $5 \times 10^6 \text{ M}^{-1}$ for haemin monomer assuming a molar difference absorptivity per haemin bound of 6.0×10^4 and a haemin self-association constant $4 \times 10^7 \text{ M}^{-1}$ (see text)

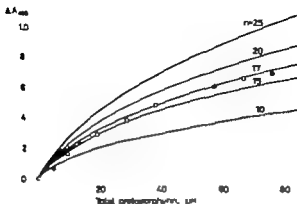


Fig. 3 Spectrophotometric titrations of spectrin at 1.6 mg/ml with protoporphyrin IX. The points refer to experimental absorbance differences at 410 nm. The curves are calculated for the indicated numbers of identical sites per chain with an intrinsic binding constant of 10^4 M^{-1} for protoporphyrin IX monomer assuming molar difference absorptivity per ligand bound of 6.9×10^4 and protoporphyrin self-association constant of $2.5 \times 10^6 \text{ M}^{-1}$ (see text).

It should be stressed that this must represent a great oversimplification, since there is no reason to believe that spectrin should contain large numbers of identical binding sites or indeed, that the sites function independently. The data would not, however, justify the use of several floating parameters to generate a best fit. The binding isotherm is in fact sufficient to describe the binding properties in operational terms.

The binding of protoporphyrin IX was studied in a similar way. The absorption spectrum generated on binding is shown in figure 1. As might be expected, it resembles that of protoporphyrin-globin [21] and protoporphyrin-albumin [15] at least in conditions of protein excess. The molar absorptivity difference on binding was estimated as 6.9×10^4 at 415 nm. In contrast to the spectrin-haemin system however the shape of the difference spectrum changes as more sites are occupied, indicating that there are at least two structurally distinct classes of site associated with absorption spectra with Soret bands at about 415 and 388 nm respectively. Again, analysis is additionally complicated by a presumed dimerisation equilibrium of the free protoporphyrin IX, with a dissociation constant of $4 \times 10^{-6} \text{ M}$ [9]. Figure 3 shows calculated curves, based on binding of the monomeric species only and on a difference absorption spectrum corresponding only to the stronger type of site. At high ligand, protein ratio, the

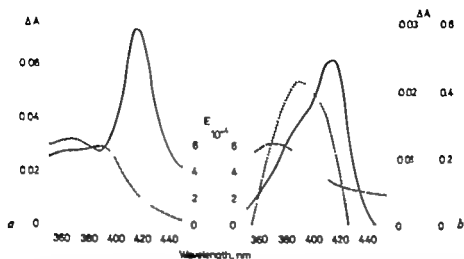


Fig 1 Spectral characteristics of haemin and protoporphyrin IX complexes of spectrin. *a* Spectrum of free haemin at pH 7.6 (---) (right-hand ordinate), and difference spectrum generated by addition of 2.25 μM haemin to spectrin at 0.43 mg/ml (—) (left-hand ordinate) *b* Spectrum of free protoporphyrin IX at pH 7.6 (---) (left hand ordinate) and difference spectra generated by addition of 1.5 μM protoporphyrin (—) and of 37 μM protoporphyrin (---) to spectrin at 0.50 mg/ml (right-hand ordinate scales, 0–0.03 and 0–0.6 respectively)

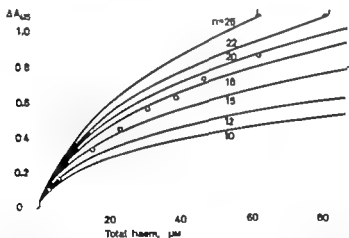


Fig 2 Spectrophotometric titrations of spectrin at 0.68 mg/ml with haemin. The points refer to experimental absorbance differences at 415 nm. The curves are calculated for the indicated numbers of identical sites per chain with an intrinsic binding constant of $5 \times 10^4 \text{ M}^{-1}$ for haemin monomer assuming a molar difference absorptivity per haemin bound of 6.0×10^4 and a haemin self-association constant $4 \times 10^4 \text{ M}^{-1}$ (see text).

the bulk of the protoporphyrin IX (and presumably of the non-haemoglobin haemin) normally present in the cell will be bound to spectrin. This will have to be considered in any description of the roles and metabolic fates of haemin and protoporphyrin IX in the cell.

Acknowledgement

We thank Mr A. C. M. HARRIS for preparations of spectrin and ghosts.

Note Added in Proof

Since submission of this paper a report by R. CAMOLLY (Evidence against the binding of native haemoglobin to spectrin of human erythrocytes. *FEBS Lett.* 85 357-360, 1978) has appeared, demonstrating by fluorescence quenching the failure of haemoglobin to bind to spectrin at physiological salt concentration; he also noted that spectrin was able to bind free haemin.

References

- 1 BEAVER, G. H., D'ALISI, A. and GRATZER, W. B. The interaction of bilirubin with human serum albumin. *Eur J Biochem.* 33 500-510 (1973).
- 2 BEAVER, G. H., CHEN, S. H., D'ALISI, A., and GRATZER, W. B. A spectroscopic study of the haemin-human serum albumin system. *Eur J Biochem.* 41 539-546 (1974).
- 3 BENOIST V. and BRANTON, D. Selective association of spectrin with the cytoplasmic surface of human erythrocyte plasma membranes. *J. biol. Chem.* 252, 2753-2762 (1977).
- 4 BURCKMEIER, W. and SCHUB, S. J. On the mechanism of ATP induced shape changes in human erythrocyte membranes II The role of ATP. *J. Cell. Biol.* 73 647-659 (1977).
- 5 BROWN, S. B., DEAN, T. C. and JONES, P. Aggregation of ferrahaems. Dimerization and protolytic equilibria of protoferrhaem and deuteroferrhaem in aqueous solution. *Biochem J* 117 733-739 (1970).
- 6 CHADMAN, P. and YUTRABONG, Y. Binding of haemoglobin to spectrin of human erythrocytes. *FEBS Lett.* 78 119-123 (1977).
- 7 DODGE, J. T., MITCHELL, C. and HANAHAN, D. J. The preparation and characteristics of hemoglobin-free ghosts of human erythrocytes. *Archs Biochem.* 100 119-130 (1963).
- 8 FISHKOP, J. H. and SMITH, K. M. Laboratory methods; in SMITH Porphyrins and metalloporphyrins (Elsevier Amsterdam 1975).
- 9 GIBSON, Q. H. The combination of porphyrins with native human globin. *J. biol. Chem.* 239 3282-3287 (1964).
- 10 GRATZER, S. B. and BEAVER, G. H. Properties of the high-molecular weight protein (spectrin) from human erythrocyte membranes. *Eur J Biochem.* 59 403-409 (1975).

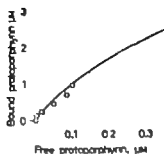


Fig 4 Binding of protoporphyrin IX to haemoglobin-free erythrocyte ghosts at 13 mg/ml (dry weight) for details see text.

calculated curves are no longer applicable in consequence of the progressive change in the absorption spectrum of the bound ligand. Only a part of the model binding isotherm can therefore be constructed. The data are sufficient however to show that there is substantial binding and that the binding isotherm may be similar to that for haemin.

Bilirubin appears not to bind to spectrin for it generates no absorption difference spectrum and no circular dichroism (cf serum albumin [1] and ligandin [5]).

Figure 4 shows part of a binding isotherm for haemoglobin-free ghosts. Again there is substantial binding in physiological conditions. This may as before be cooperative in character but we have been unable to obtain data of sufficient precision to allow a more detailed analysis of the binding.

Sedimentation velocity experiments at approximately physiological ionic strength (see above) give no evidence of interaction between spectrin and haemoglobin. The spectrin preparations show the major boundary of the dimeric form sedimenting at 9.9 S at the concentration used with a small proportion of more rapidly migrating tetramer [cf ref 10, 14]. Added haemoglobin sediments independently. The binding observed by CHAIMANEE and YUTHAVONG [6] thus does not occur in physiological conditions. It is evidently a non-specific, electrostatically dominated interaction which is suppressed by added electrolyte.

The concentration of protoporphyrin IX normally found in the erythrocyte is in the micromolar range [19] but is much higher in some pathological conditions and also in reticulocytes. Free haemin plays an important role in controlling the rate of biosynthesis of haemoglobin in reticulocytes but its normal concentration in the cell appears not to be known. It must be expected from the binding isotherms shown here that

the bulk of the protoporphyrin IX (and presumably of the non-haemoglobin haemin) normally present in the cell will be bound to spectrin. This will have to be considered in any description of the roles and metabolic fates of haemin and protoporphyrin IX in the cell.

Acknowledgement

We thank M. A. C. M. HARRIS for preparations of spectrin and ghosts.

Note Added in Proof

Since submission of this paper a report by R. CARROLL (Evidence against the binding of native haemoglobin to spectrin of human erythrocytes *FEBS Lett.* **85** 357-360, 1978) has appeared, demonstrating by fluorescence quenching the failure of haemoglobin to bind to spectrin at physiological salt concentration, he also noted that spectrin was able to bind free haemin.

References

- 1 BEA TH, G. H., D ALBA, A., and GRATZER, W. B. The interaction of bilirubin with human serum albumin. *Eur J Biochem.* **33** 500-510 (1973).
- 2 BEA TH, G. H., CHEN, S. H., D ALBA, A., and GRATZER, W. B. A spectroscopic study of the haemin-human serum albumin system. *Eur J Biochem.* **41** 539-546 (1974).
- 3 BENNETT, V. and BRANTON, D. Selective association of spectrin with the cytoplasmic surface of human erythrocyte plasma membranes. *J biol. Chem.* **252** 2753-2762 (1977).
- 4 BRIDGMAN, W. and SINGER, S. J. On the mechanism of ATP induced shape changes in human erythrocyte membranes II The role of ATP. *J Cell Biol.* **73** 647-659 (1977).
- 5 BROWN, S. B., DEAN, T. C. and JONES, B. Aggregation of ferrihaem. Dimerization and protolytic equilibria of protoferrihaem and deuterioferrihaem in aqueous solution. *Biochem J* **117** 733-739 (1970).
- 6 CHADMAN, P. and YUTHA-ONG, Y. Binding of haemoglobin to spectrin of human erythrocytes. *FEBS Lett.* **78** 119-123 (1977).
- 7 DODGE, J. T., MITCHELL, C. and HANAHAN, D. J. The preparation and characteristics of haemoglobin free ghosts of human erythrocytes. *Archs Biochem.* **100** 119-130 (1963).
- 8 FURUBO, J. H. and SMITH, K. M. Laboratory methods. *SMITH Porphyrins and metalloporphyrins* (Elsevier Amsterdam 1975).
- 9 ODMON, Q. H. The combination of porphyrins with native human globin. *J biol. Chem.* **239** 3282-3287 (1964).
- 10 GRATZER, S. B. and BEA TH, G. H. Properties of the high-molecular weight proteins (spectrin) from human erythrocyte membranes. *Eur J Biochem.* **58** 403-409 (1975).

- 11 HITCHCOCK, S. E. Regulation of cell motility in nonmuscle cells. *J Cell Biol* 74 1-15 (1977)
- 12 INADA, Y. and SHIBATA, K. The Soret band of monomeric hematin and its changes on polymerization. *Biochem. biophys. Res. Commun.* 9 323-327 (1964)
- 13 JULIANO R. L. KIMELBERG H. K., and PAPAHADJOPOULOS, D. Synergistic effects of a membrane protein (spectrin) and Ca on the Na permeability of phospholipid vesicles. *Biochim. biophys. Acta* 241 894-905 (1971)
- 14 KAM, Z. JOSEPH, R. EISENBERG, H. and GRATZER, W. B. Structural study of spectrin from human erythrocyte membranes. *Biochemistry* 16. 5568-5572 (1977).
- 15 MACILLY A. C. Haemin-protein binding in peroxidase and methaemalbumin. *Nature Lond.* 192 630-633 (1961)
- 16 MARCHESI, V. T. Isolation of spectrin from erythrocyte membranes. *Meth. Enzym.* vol. 32, pp 275-277 (Academic Press, New York 1974)
- 17 MARCHESI, V. T. FURTHMAYER, H. and TOMITA, M. The red cell membrane. *A. Rev. Biochem.* 45 667-698 (1976).
- 18 PENNELL, B. R. Composition of normal human red cells, in BRISTOW and SURGEON. *The red blood cell* pp 29-69 (Academic Press, New York 1964)
- 19 PINDER, J. C. BRAY D. and GRATZER, W. B. Control of the interaction of spectrin and actin by phosphorylation. *Nature, Lond.* 270 752-754 (1977).
- 20 ROSENTHAL, A. S. KREONOW F. M. and MOSES, H. L. Some characteristics of a Ca⁺⁺-dependent ATPase activity associated with a group of erythrocyte membrane proteins, which form fibrils. *Biochim. biophys. Acta* 196 254-262 (1970).
- 1 ROSS-FANELLI, A. ANTONINI E., and CAPUTO, A.. Studies on the structure of hemoglobin. II Properties of reconstituted protohemoglobin and protoporphyrin-globin. *Biochim. biophys. Acta* 35 93-101 (1959).
- 22 SCATCHARD G. The attraction of proteins for small molecules and ions. *Ann. N.Y. Acad. Sci.* 51 660-672 (1949)
- 23 STECK, T. L. The organization of proteins in the human red blood cell membrane. *J Cell Biol.* 62 1-19 (1974).
- 24 SWEET C. and ZULL, J. E. Interaction of the erythrocyte membrane protein spectrin with model membrane systems. *Biochem. biophys. Res. Commun.* 41 135-141 (1970).
- 25 TIPPING, E. KETTERER, B. CHRISTODOULIDES, L., and ENDERBY G. Spectroscopic studies of the binding of bilirubin by ligandin and aminoazo-dye-binding protein A. *Biochem. J* 157 211-216 (1976)
- 6 TIPPING, E. KETTERER, B. CHRISTODOULIDES, L., and ENDERBY G. The interactions of haem with ligandin and aminoazo-dye-binding protein A. *Biochem. J* 157 461-467 (1976)
- 27 WANG, K. and RICHARDS, F. M. An approach to nearest neighbor analysis of membrane proteins. Application to the human erythrocyte membrane of a method employing cleavable cross-links. *J. Biol. Chem.* 249 8005-8018 (1974)

Studies on Red Cell Flexibility in Spherocytosis Using a Polycarbonate Membrane Filtration Method

R. JOHNNSSON and P. VUOMO

Department of Clinical Chemistry, University of Helsinki and IIrd Department of Medicine, Helsinki University Central Hospital, Meilahti Hospital, Helsinki

Key Words. Red cell flexibility Spherocytosis Vincristine Chlorpromazine

Abstract. A method for studying red cell flexibility as reflected by red cell rigidity and fragility is described. Using an infusion pump, suspended red cells are filtered through polycarbonate membrane with 3 μ m pores. The filtration pressure, which is continuously monitored, is considered to reflect red cell rigidity. The hemoglobin released by the disruption of red cells passing through the membrane is regarded as an indirect measure of red cell fragility. In all of the patients with hereditary spherocytosis studied and in some of their symptomatic relatives, the decreased flexibility observed was associated with increased rigidity of the red cell. *In vitro* effects of chlorpromazine and vinca alkaloid on red cell flexibility were also studied. High concentrations of chlorpromazine and vincristine induced formation of spherocytes displaying altered cell flexibility.

Erythrocyte flexibility has been studied monitoring red cell filtration through paper [24], various porous membranes [7-22] and silver filters [1] with the filtration time being used as a measure of the flexibility of the red cell membrane. The pressure needed to transfer erythrocytes through a micropipette has also been employed to assess red cell flexibility [14] as has the viscosity of the red cell suspension, especially in studying poikilocytes [5]. In all these approaches alterations in red cell viscoelasticity are reflected by one parameter only and, because of this, it has been difficult to elucidate the pathophysiological mechanism of the altered flexibility. This paper describes a method which provides a measure of both the rigidity and fragility of the red cell. The rigidity is estimated from pressure readings obtained using a manometer connected to an infusion pump.

- 11 HITCHCOCK, S. E. Regulation of cell motility in nonmuscle cells. *J Cell Biol* 74 1-15 (1977).
- 12 INADA, Y. and SHIBATA, K. The Soret band of monomeric hematin and its changes on polymerization. *Biochem. biophys. Res. Commun.* 9 323-327 (1962).
- 13 JULIANO R. L., KIMELBERG, H. K., and PAPAHADJOPOULOS, D. Synergistic effects of a membrane protein (spectrin) and Ca^{++} on the Na^+ permeability of phospholipid vesicles. *Biochim. biophys. Acta* 241 894-905 (1971).
- 14 KAM, Z., JOSEPH, R., EISENBERG, H., and GRATZER, W. B. Structural study of spectrin from human erythrocyte membranes. *Biochemistry* 16 5568-5572 (1977).
- 15 MACFELY, A. C. Haemin-protein binding in peroxidase and methaemalbumin. *Nature Lond.* 192 630-633 (1961).
- 16 MARCHESI, V. T. Isolation of spectrin from erythrocyte membranes. *Meth. Enzym.* vol 32, pp. 275- 77 (Academic Press, New York 1974)
- 17 MARCHESI, V. T., FURTHMAYER, H. and TOMITA, M. The red cell membrane. *A. Rev Biochem.* 45 667-698 (1976)
- 18 PENNELL, B. R. Composition of normal human red cells, in BISHOP and SURGEON. *The red blood cell* pp 29-69 (Academic Press, New York 1964).
- 19 PINDER, J. C., BRAY D. and GRATZER, W. B. Control of the interaction of spectrin and actin by phosphorylation. *Nature, Lond.* 270 75 -754 (1977).
- 20 ROSENTHAL, A. S., KREGENOW, F. M. and MOSES, H. L. Some characteristics of a Ca^{++} -dependent ATPase activity associated with a group of erythrocyte membrane proteins, which form fibrils. *Biochim. biophys. Acta* 196. 254-262 (1970)
- 1 ROSSI FANELLI, A., ANTONINI, E., and CAPUTO, A. Studies on the structure of hemoglobin. II Properties of reconstituted protohemoglobin and protoporphyrin globin. *Biochim. biophys. Acta* 35 93-101 (1959).
- 22 SCATCHARD, G. The attraction of proteins for small molecules and ions. *Ann. NY Acad. Sci* 51 660-672 (1949)
- 23 STECK, T. L. The organization of proteins in the human red blood cell membrane. *J Cell Biol.* 62 1-19 (1974).
- 24 SWEET, C. and ZULL, J. E. Interaction of the erythrocyte membrane protein, spectrin, with model membrane systems. *Biochem. biophys. Res. Commun.* 41 135-141 (1970).
- 25 TIPPING, E., KETTERER, B., CHRISTODOULIDES, L., and ENDERBY, G. Spectroscopic studies of the binding of bilirubin by ligandin and aminoazo-dye-binding protein A. *Biochem. J* 157 11-216 (1976).
- 6 TIPPING, E., KETTERER, B., CHRISTODOULIDES, L., and ENDERBY, G. The interactions of haem with ligandin and aminoazo-dye-binding protein A. *Biochem. J* 157 461-467 (1976)
- 27 WANG, K. and RICHARDS, F. M. An approach to nearest neighbor analysis of membrane proteins. Application to the human erythrocyte membrane of a method employing cleavable cross-links. *J Biol Chem.* 249 8005-8018 (1974).

Studies on Red Cell Flexibility in Spherocytosis Using a Polycarbonate Membrane Filtration Method

R. JOHNNSSON and P. VUORIO

Department of Clinical Chemistry University of Helsinki and Third Department
of Medicine, Helsinki University Central Hospital, Meilahti Hospital, Helsinki

Key Words. Red cell flexibility Spherocytosis Vinblastine Chlorpromazine

Abstract. A method for studying red cell flexibility as reflected by red cell rigidity and fragility is described. Using an infusion pump, suspended red cells are filtered through a polycarbonate membrane with 3 μ m pores. The filtration pressure, which is continuously monitored, is considered to reflect red cell rigidity. The hemoglobin released by the disruption of red cells passing through the membrane is regarded as an indirect measure of red cell fragility. In all of the patients with hereditary spherocytosis studied and in some of their symptomless relatives, the decreased flexibility observed was associable with increased rigidity of the red cell. The effects of chlorpromazine and vaso alkaloid on red cell flexibility were also studied. High concentrations of chlorpromazine and vinblastine induced formation of spherocytes displaying altered cell flexibility.

Erythrocyte flexibility has been studied monitoring red cell filtration through paper [24] various porous membranes [7-22] and silver filters [1] with the filtration time being used as a measure of the flexibility of the red cell membrane. The pressure needed to transfer erythrocytes through a micropipette has also been employed to assess red cell flexibility [14] as has the viscosity of the red cell suspension, especially in studying polkylo-cytes [5]. In all these approaches alterations in red cell viscoelasticity are reflected by one parameter only and, because of this, it has been difficult to elucidate the pathophysiological mechanism of the altered flexibility. This paper describes a method which provides a measure of both the rigidity and fragility of the red cell. The rigidity is estimated from pressure readings obtained using a manometer connected to an infusion pump-

- 11 HITCHCOCK, S. E. Regulation of cell motility in nonmuscle cells. *J Cell Biol.* 74 1-15 (1977)
- 12 INADA, Y. and SHIMATA, K. The Soret band of monomeric hematin and its changes on polymerization. *Biochem biophys. Res. Commun.* 9 323-327 (1962)
- 13 JULIANO R. L., KIBELBERG H. K., and PAPAIOPOULOS, D. Synergistic effects of a membrane protein (spectrin) and Ca on the Na permeability of phospholipid vesicles. *Biochim. biophys. Acta* 241 894-905 (1971)
- 14 KAM, Z., JOSEPHS, R., EISENBERG, H. and GRATZER, W. B. Structural study of spectrin from human erythrocyte membranes. *Biochemistry* 16, 5568-5577 (1977)
- 15 MAERLY, A. C. Haemlin protein binding in peroxidase and methaemalbumin. *Nature, Lond.* 192 630-633 (1961)
- 16 MARCHESI, V. T. Isolation of spectrin from erythrocyte membranes. *Meth. Enzym.* vol 32, pp 275-277 (Academic Press, New York 1974)
- 17 MARCHESI, V. T., FURTHMAYER, H. and TORITTA, M. The red cell membrane. *A. Rev Biochem.* 45 667-698 (1976)
- 18 PENNELL, B. R. Composition of normal human red cells in BISHOP and SURGEON. *The red blood cell*, pp 29-69 (Academic Press, New York 1964).
- 19 PINDER, J. C., BRAY D. and GRATZER, W. B. Control of the interaction of spectrin and actin by phosphorylation. *Nature, Lond.* 270 752-754 (1977)
- 20 ROSENTHAL, A. S., KROENOW P. M. and MOSES, H. L. Some characteristics of a Ca^{++} -dependent ATPase activity associated with a group of erythrocyte membrane proteins, which form fibrils. *Biochim. biophys. Acta* 196, 254-262 (1970)
- 21 ROSSI FANELLI, A., ANTONINI, E., and CAPUTO, A. Studies on the structure of hemoglobin. II Properties of reconstituted protohemoglobin and protoporphyrin-globin. *Biochim. biophys. Acta* 35 93-101 (1959).
- 22 SCATCHARD G. The attraction of proteins for small molecules and ions. *Ann. N.Y. Acad. Sci.* 51 660-672 (1949).
- 23 STECK, T. L. The organization of proteins in the human red blood cell membrane. *J Cell Biol.* 62 1-19 (1974)
- 24 SWEET C. and ZULL, J. E. Interaction of the erythrocyte membrane protein, spectrin, with model membrane systems. *Biochem. biophys. Res. Commun.* 41 135-141 (1970)
- 25 TIPPING E., KETTERER, B., CHRISTODOULIDES, L., and ENDERBY G. Spectroscopic studies of the binding of bilirubin by ligandin and aminoazo-dye-binding protein A. *Biochem. J* 157 211-216 (1976).
- 26 TIPPING, E., KETTERER, B., CHRISTODOULIDES, L., and ENDERBY G. The interactions of haem with ligandin and aminoazo-dye-binding protein A. *Biochem. J* 157 461-467 (1976)
- 27 WANG, K. and RICHARDS, F. M. An approach to nearest neighbor analysis of membrane proteins. Application to the human erythrocyte membrane of a method employing cleavable cross-links. *J. biol. Chem.* 249 8005-8018 (1974)

quot of unfiltered blood was simultaneously centrifuged to detect possible autohemolysis and to serve as a "blank". Hemoglobin was measured by the benzidine method [20]. Benzidine was obtained from Fluka AG, Buchs, Switzerland.

Membranes from several lots were inspected microscopically and the diameter of the pores measured from photographs taken prior to use. The pore size was quite similar in membranes from the same lot, but inter-lot variations were observed. Because of this, membranes from new lots were tested with blood from control subjects prior to use in studies on HS patients and drug-treated red cells. The membranes were not reused.

Results

Control Experiments

The injection velocity was kept constant at all blood cell concentrations tested including packed red cells. Using an injection velocity of 0.5 ml/min, no marked red cell damage was observed when filtering a 10% suspension of normal erythrocytes within 1 h of venipuncture.

The hemoglobin concentration in the supernatant of the "blank" was subtracted from that of the filtered sample and the difference was called the 'free hemoglobin concentration'. The 'free hemoglobin in 50 experiments on red cells from control subjects was 54 ± 27 mg/l (mean \pm SD).

At the beginning of the filtration procedure there were pressure fluctuations due to the sensitivity of the pressure transducer but after 1-2 ml had passed through the filter the increase in pressure was smooth. The pressure curve was always quite linear between 2 and 5 ml. The maximal pressure in 30 control experiments was 101 ± 30 mm Hg (mean \pm SD). The distributions of 'free hemoglobin and maximal pressure values were approximately normal in the control group.

The precision of the method was studied in duplicate experiments performed on the same day within 1 h, using blood from control subjects. The coefficient of variation (CV %) for the pressure measurements was 9.7 ($n = 28$) and for the 'free hemoglobin determinations 11.5 ($n = 23$). Time to-time variability was also examined in control subjects restudied on a second occasion. The time interval varied from 2 days to 12 months and CV for the pressure measurements was 16.1 ($n = 9$) and for the 'free hemoglobin 16.7 ($n = 16$).

Hereditary Spherocytosis

The values for 'free hemoglobin in the HS patients and in 4 of their relatives are presented in table I. Although the degree of red cell damage in the same HS patient varied from time to time (all measurements were

membrane device. Fragility is reflected by the degree of hemolysis induced by forcing the red cells through the porous polycarbonate membrane. The method described is rapid and the required apparatus can be constructed from ordinary laboratory equipment. Using this system the flexibility of the erythrocytes from patients with congenital or hereditary spherocytosis (HS) and their relatives has been studied. The *in vitro* effects of vinblastine and chlorpromazine on red cell flexibility were also studied as these drugs have been reported to change the osmotic fragility and cellular morphology producing red cells resembling spherocytes from HS patients [10].

Material and Methods

Material

Venous blood from healthy persons aged 20-75 years was collected into tubes containing 100 U of heparin/ml blood. Blood cells were counted in a Coulter Counter Model S (Coulter Electronics Inc., Hialeah, Fla.) The blood was diluted with 0.9% NaCl to give varying concentrations of red cells and a 10% suspension was used in the final experiments. Packed red cells (cell percent about 95) were used to check the stability of the polycarbonate membranes.

Blood samples from 13 HS patients from 7 families were tested. 11 of these patients had been splenectomized previously. 22 relatives of the HS patients were also studied. 4 of these each from a different family yielded abnormal results which are presented with the HS group.

Vinblastine sulfate (Velbe®) was obtained from Eli Lilly and Company (Indianapolis, Ind.) and chlorpromazine chloride (Largactil®) from Medica, Finland.

Methods

Osmotic resistance was measured using hypotonic saline [3].

The drug-induced morphological changes in the erythrocytes were studied microscopically in wet preparations and after fixation and staining with May-Grünwald-Giemsa [3].

Filtration Technique. A 5.0-ml blood sample was drawn into a syringe which matched the holders of the infusion pump. The sample was forced through a polycarbonate filter (pore diameter 3 μ m Shandon Labortechnik GmbH Frankfurt, FRG) using an infusion pump (Type 1833, Braun Apparatenbau, Melsungen, FRG) fixed in upright position. The pressure of the blood suspension was continuously registered during the injection by a pressure transducer (Statham P 23 I a, Kulite Sensors Limited Hampshire, England) inserted between the syringe and the membrane holder (diameter 13 mm, Pop Top, Nuclepore Corporation, Pleasanton, Calif.) A pressure amplifier (Olli 223 Ollituote Oy Kivenlahti, Finland) was used for monitoring the changes in filtration pressure.

The filtered sample was centrifuged at 1,000 g for 10 min and an aliquot of the supernatant was removed for measurement of released hemoglobin. A similar all

quot of unfiltered blood was simultaneously centrifuged to detect possible autohemolysis and to serve as 'blank'. Hemoglobin was measured by the benzidine method [20]. Benzidine was obtained from Fluka AG, Buchs, Switzerland.

Membranes from several lots were inspected microscopically and the diameter of the pores measured from photographs taken prior to use. The pore size was quite smaller in membranes from the same lot, but inter-lot variations were observed. Because of this, membranes from new lots were tested with blood from control subjects prior to use in studies on HS patients and drug-treated red cells. The membranes were not reused.

Results

Control Experiments

The injection velocity was kept constant at all blood cell concentrations tested including packed red cells. Using an injection velocity of 0.5 ml/min, no marked red cell damage was observed when filtering a 10% suspension of normal erythrocytes within 1 h of venipuncture.

The hemoglobin concentration in the supernatant of the 'blank' was subtracted from that of the filtered sample and the difference was called the 'free hemoglobin concentration'. The free hemoglobin in 50 experiments on red cells from control subjects was 5.4 ± 2.7 mg/l (mean \pm SD).

At the beginning of the filtration procedure there were pressure fluctuations due to the sensitivity of the pressure transducer but after 1-2 ml had passed through the filter the increase in pressure was smooth. The pressure curve was always quite linear between 2 and 5 ml. The maximal pressure in 30 control experiments was 101 ± 30 mm Hg (mean \pm SD). The distributions of 'free hemoglobin and maximal pressure values were approximately normal in the control group.

The precision of the method was studied in duplicate experiments performed on the same day within 1 h, using blood from control subjects. The coefficient of variation (CV %) for the pressure measurements was 9.7 (n = 28) and for the 'free hemoglobin determinations 11.5 (n = 23). Time-to-time variability was also examined in control subjects restudied on a second occasion. The time interval varied from 2 days to 12 months and CV for the pressure measurements was 16.1 (n = 9) and for the 'free hemoglobin 16.7 (n = 16).

Hereditary Spherocytosis

The values for 'free hemoglobin in the HS patients and in 4 of their relatives are presented in table I. Although the degree of red cell damage in the same HS patient varied from time to time (all measurements were

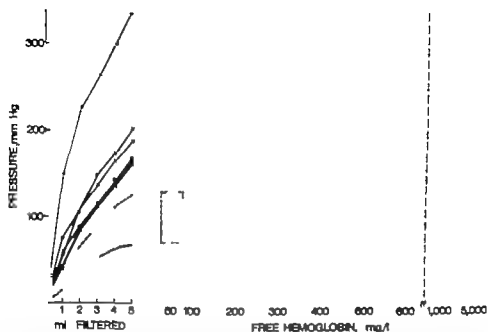


Fig 1 Blood sample filtration experiments. The left side presents the range of the pressure versus filtered volume (1-5 ml) when studying HS patients (●) and 4 of their relatives (×). The right side demonstrates the maximal pressure versus free hemoglobin concentration in each of these subjects. The dark fields represent the corresponding control results (mean \pm SD).

performed in the space of 1 year) the free hemoglobin was always much higher than in any control subject studied. Patient III 1 had a normal blood hemoglobin concentration even before splenectomy and only 2% spherocytes but the high pressure observed during filtration and the marked disruption of the red cells indicated reduced cell flexibility in this case also. The maximal pressures registered during filtration of HS blood samples are also presented in table I. Because the pressure device was introduced after studies on some HS patients had begun pressure recordings are lacking in several of the first measurements.

The 4 close relatives of individual HS patients who displayed abnormal flexibility all had normal blood counts and osmotic resistance. More extensive hematological investigations were carried out on subjects V 2 and VI 2 than in the 2 others. In both of these subjects the red cell survival time and serum haptoglobin were marginally decreased which were the only features suggesting hemolytic anemia. In the 2 other relatives studied, the inheritance of the disease was most probable because of the distri-

Table 1. Free hemoglobin and maximal pressure in Nuclepore membrane filtration experiments on blood samples from HS patients and their relatives

Subject symbol	Free hemoglobin, mg/l/Pressure, mm Hg	
	first measurement	second measurement
<i>Patient</i>		
I-1	6,307/292	
I-2	809	
II-1	2,199	3,630/336
II-2	948	1,764
II-3	952	1,753/204
III 1	3,623/228	
IV 1	840	
IV-2	860	4,429/189
IV 3	1,404	8,013/197
IV-4	2,800	440, 164
V 1	606	1,920/196
VI-1	4,840	4,799/305
VII-1	359	
<i>Relatives</i>		
III-2	529/187	
IV 5	376	146/202
V 2	88/161	
VI-2	220	36/166
<i>Controls</i>		
Mean (\pm S.D.)	57 \pm 27/101 \pm 30	
	50	30

Not splenectomized.

bution and frequency of the disease in their family. Their red cell fragility scores were similar to those of the patients with manifest HS disease. From a second measurement on subject IV 5 a result resembling that seen in subjects V 2 and VI 2 was obtained. These results are summarized in figure 1.

In vitro Induction of Spherocyte Formation

Experiments with Vinca Alkaloid Red cells from 7 control subjects were incubated in 23 experiments with varying concentration of vinblastine. At concentrations of 3–200 μ mol/l no constant effect on either rigidity or

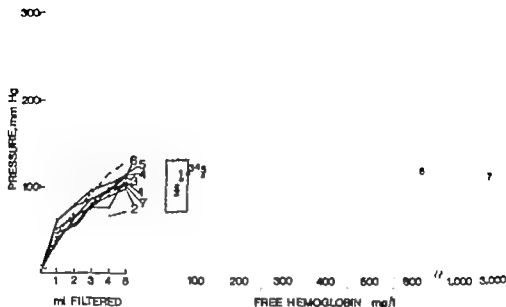


Fig 2 Blood sample filtration experiments. The left side presents the mean pressure versus filtered volume (1–5 ml) when studying normal blood samples treated with various concentrations of vinblastine. The right side demonstrates the mean maximal pressure versus mean free hemoglobin concentration in these treated samples. The dark fields represent the corresponding control results (mean \pm SD). 1 = 3, 2 = 5, 3 = 10, 4 = 200, 5 = 280, 6 = 400 and 7 = 550 μ mol/l vinblastine. Each point represents the mean of 3–4 experiments.

fragility was found. With concentrations over 280 μ mol/l increased fragility was observed but only at the highest concentrations tested was there always marked disruption of the red cells (fig. 2). The pressure increase relative to free hemoglobin concentration was, however, less than that observed in HS patients. In these experiments vinblastine concentrations up to 280 μ mol/l did not significantly alter the osmotic resistance of the red cells. At higher concentrations of this drug the osmotic resistance decreased with increasing concentrations.

The NaCl induced echinocytes (fig. 3a) became smoother already in the presence of small concentrations of vinblastine. With higher concentrations of this drug the red cells took the form of stomatocytes and in the presence of very high concentrations there were also spherocytes (fig. 3b).

Experiments with Chlorpromazine Red cells from 6 control subjects were treated with 7–200 μ mol/l of chlorpromazine. The mean pressure curves and the free hemoglobin versus pressure recorded are shown in



Fig. 3 NaCl-induced echinocytes. *a* Erythrocytes treated with 550 μ mol/l vinblastine. Erythrocytes treated with 200 μ mol/l chlorpromazine. May-Grünwald-Giemsa. $\times 400$.

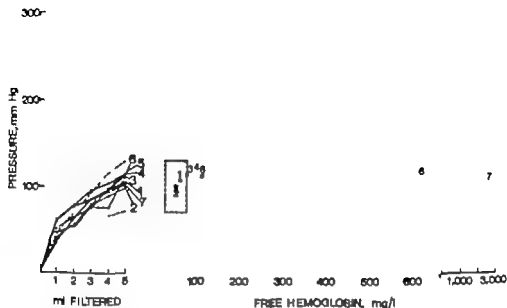


Fig 2 Blood sample filtration experiments. The left side presents the mean pressure versus filtered volume (1–5 ml) when studying normal blood samples treated with various concentrations of vinblastine. The right side demonstrates the mean maximal pressure versus mean 'free hemoglobin concentration in these treated samples. The dark fields represent the corresponding control results (mean \pm SD). 1 = 7 2 = 5 3 = 10, 4 = 200 5 = 280, 6 = 400 and 7 = 550 $\mu\text{mol/l}$ vinblastine. Each point represents the mean of 3–4 experiments.

fragility was found. With concentrations over 280 $\mu\text{mol/l}$ increased fragility was observed but only at the highest concentrations tested was there always marked disruption of the red cells (fig. 2). The pressure increase relative to free hemoglobin concentration was, however, less than that observed in HS patients. In these experiments vinblastine concentrations up to 280 $\mu\text{mol/l}$ did not significantly alter the osmotic resistance of the red cells. At higher concentrations of this drug the osmotic resistance decreased with increasing concentrations.

The NaCl-induced echinocytes (fig 3a) became smoother already in the presence of small concentrations of vinblastine. With higher concentrations of this drug the red cells took the form of stomatocytes and in the presence of very high concentrations there were also spherocytes (fig 3b).

Experiments with Chlorpromazine Red cells from 6 control subjects were treated with 7–200 $\mu\text{mol/l}$ of chlorpromazine. The mean pressure curves and the 'free hemoglobin versus pressure recorded are shown in

served significant variation in the pore size of membranes from different lots and were therefore compelled to test membranes from new lots prior to use. BAAR [1] who used silver membranes, did not report encountering such problems of methodological reproducibility but unlike the polycarbonate membranes, the silver membranes were reused and had therefore to be washed and regenerated making the procedure quite tedious. Furthermore, the irregular pore form and the somewhat varying pore size of the silver membranes must also be regarded as disadvantageous.

Earlier methods for assessing red cell flexibility did so only on the basis of a single parameter [1 7 14 22, 24]. This is often insufficient for establishing whether red cell rigidity or fragility or both, is implicated. As an abnormal red cell flexibility in HS is well documented [2, 17] we elected to use erythrocytes from HS patients to validate the method. In all the HS samples tested the increased rigidity of the red cell was reflected by pressure readings higher than those observed in the controls. Increased red cell rigidity was also noted in a HS patient whose erythrocytes lacked the typical spheropoikilocytosis. The clinical course of HS is characterized by varying degrees of hemolysis (aplastic and hyperplastic crises). Removal of the spleen, however eliminates one of the provocative factors in red cell destruction, although it does not abolish the membrane defect [2]. This would agree with our observations of a considerable variation from time to time in red cell flexibility in HS patients.

Of the hematologically normal relatives studied, 4 had abnormally inflexible red cells as reflected by either increased "free" hemoglobin and/or increased pressure readings. Preliminary observations in this laboratory on the osmotic resistance of their red cells using an ouabain sensitization test and on the red cell Ca^{++} -transport yielded results resembling those found for the HS group. In these 4 symptomless relatives the increase in filtration pressure was of a magnitude similar to that seen in the HS patients. However the membrane abnormality in the relatives was evidently not quite as severe as less red cell fragmentation was observed.

A number of contractile filamentous proteins have been found in the red cell membrane. Of these proteins, spectrin, which is also called myosin-like protein, and a smaller actin-like protein are the best characterized [15-18]. The changes in red cell membranes in HS have been explained with changes in spectrin [11].

Vinblastine, a drug which precipitates filamentous proteins, has been reported to act mainly on spectrin [1] and to transform normal red cells into spherocytes. According to JACOB *et al.* [10-12] these spherocytes are

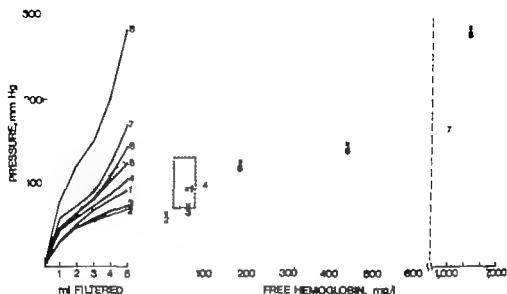


Fig 4 Blood sample filtration experiments. The left side presents the mean pressure versus filtered volume (1-5 ml) when studying normal blood samples treated with various concentrations of chlorpromazine. The right side demonstrates the mean maximal pressure versus mean free hemoglobin concentration in these treated samples. The dark fields represent the corresponding control values (mean \pm SD) 1 = 7 2 = 15 3 = 35 4 = 50, 5 = 70, 6 = 100, 7 = 150 and 8 = 200 μ mol/l chlorpromazine. Each point represents the mean of 3-6 experiments.

figure 4 Drug induced fragility of the red cells was observed to start at a chlorpromazine concentration of 50 μ mol/l. Osmotic resistance was also measured in the chlorpromazine treated red cells at a concentration of 7 μ mol/l the osmotic resistance was increased while at chlorpromazine concentrations greater than 50 μ mol/l it decreased with increasing concentrations

In the presence of low concentrations of chlorpromazine the echinocytes were transformed into discocytes. With increasing drug concentrations the spherocytes become more numerous and at the highest concentration tested only spherocytes were observed (fig. 3c)

Discussion

The polycarbonate membrane has previously been found suitable for studying red cell flexibility [7 22] However in the present study we ob-

served significant variation in the pore size of membranes from different lots and were therefore compelled to test membranes from new lots prior to use. BAAR [1] who used silver membranes, did not report encountering such problems of methodological reproducibility but unlike the polycarbonate membranes, the silver membranes were reused and had therefore to be washed and regenerated making the procedure quite tedious. Furthermore, the irregular pore form and the somewhat varying pore size of the silver membranes must also be regarded as disadvantages.

Earlier methods for assessing red cell flexibility did so only on the basis of a single parameter [1 7 14 22, 24] This is often insufficient for establishing whether red cell rigidity or fragility or both, is implicated. As an abnormal red cell flexibility in HS is well documented [2, 17] we elected to use erythrocytes from HS patients to validate the method. In all the HS samples tested the increased rigidity of the red cell was reflected by pressure readings higher than those observed in the controls. Increased red cell rigidity was also noted in a HS patient whose erythrocytes lacked the typical spheropoikilocytosis. The clinical course of HS is characterized by varying degrees of hemolysis (aplastic and hyperplastic crises). Removal of the spleen, however eliminates one of the provocative factors in red cell destruction, although it does not abolish the membrane defect [2] This would agree with our observations of a considerable variation from time to time in red cell flexibility in HS patients.

Of the hematologically normal relatives studied, 4 had abnormally inflexible red cells as reflected by either increased free hemoglobin and/or increased pressure readings. Preliminary observations in this laboratory on the osmotic resistance of their red cells using an ouabain sensitization test and on the red cell Ca^{++} transport yielded results resembling those found for the HS group. In these 4 symptomless relatives the increase in filtration pressure was of a magnitude similar to that seen in the HS patients. However the membrane abnormality in the relatives was evidently not quite as severe as less red cell fragmentation was observed.

A number of contractile filamentous proteins have been found in the red cell membrane. Of these proteins, spectrin, which is also called myosin-like protein, and a smaller actin-like protein are the best characterized [15 18] The changes in red cell membranes in HS have been explained with changes in spectrin [11]

Vinblastine, a drug which precipitates filamentous proteins, has been reported to act mainly on spectrin [12] and to transform normal red cells into spherocytes. According to JACOB *et al.* [10-1] these spherocytes are

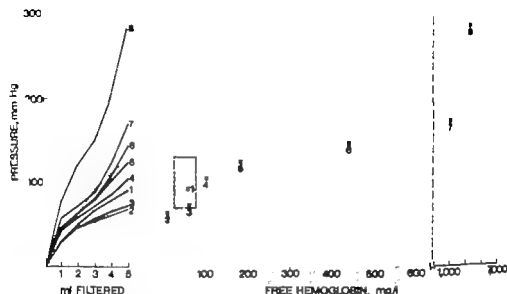


Fig 4 Blood sample filtration experiments. The left side presents the mean pressure versus filtered volume (1–5 ml) when studying normal blood samples treated with various concentrations of chlorpromazine. The right side demonstrates the mean maximal pressure versus mean free hemoglobin concentration in these treated samples. The dark fields represent the corresponding control values (mean \pm SD). 1 = 7 2 = 15 3 = 35 4 = 50 5 = 70 6 = 100, 7 = 150 and 8 = 200 $\mu\text{mol/l}$ chlorpromazine. Each point represents the mean of 3–6 experiments.

figure 4 Drug induced fragility of the red cells was observed to start at a chlorpromazine concentration of 50 $\mu\text{mol/l}$. Osmotic resistance was also measured in the chlorpromazine treated red cells at a concentration of 7 $\mu\text{mol/l}$ the osmotic resistance was increased while at chlorpromazine concentrations greater than 50 $\mu\text{mol/l}$ it decreased with increasing concentrations.

In the presence of low concentrations of chlorpromazine the echinocytes were transformed into discocytes. With increasing drug concentrations the spherocytes become more numerous and at the highest concentration tested only spherocytes were observed (fig 3c)

Discussion

The polycarbonate membrane has previously been found suitable for studying red cell flexibility [7 22] However in the present study we ob-

meaningfulness of this approach was highlighted by demonstration of differences in the flexibility of drug-induced spherocytes and erythrocytes from HS patients, while in previous studies using single parameter methods such differences could not be detected. This applies, in particular to comparative studies on vinblastine treated red cells and spherocytes from HS patients. The present method would also seem useful for detecting abnormalities in red cell flexibility in symptomatic relatives of HS patients.

Acknowledgement

Our thanks are due to Dr ANJA-LIISA KARELTO, PhD, for valuable technical advice. This study was supported by grants from the Finnish Heart Research Foundation.

References

- 1 BAAR, S.: A convenient and reproducible filtration technique for the determination of erythrocyte flexibility *Br J Haemat.* 34 69-78 (1976).
- 2 DACE, J. V. Hereditary spherocytosis; in *The haemolytic anemias, congenital and acquired*, pp. 49-93 (Churchill, London 1954).
- 3 DACE, J. V. and LEWIS, S. M. *Practical haematology* pp. 166-171 (Churchill, London 1968).
- 4 DEUTCHER, B. Transformation and restoration of biconcave shape of human erythrocytes induced by amphiphilic agents and changes of ionic environment. *Biochim. biophys. Acta* 163 494-500 (1968).
- 5 DREWETZ, L. Rheology of blood in *Diagnostic and preventive medicine. An introduction to clinical hemorheology* pp. 203-220 (Butterworths, London 1976).
- 6 FENG, S. A. and BASKILL, S. Increased erythrocyte Ca^{++} -content in hereditary spherocytosis. *Pediat. Res.* 9 928-931 (1975).
- 7 GERSHBERG, M. I., BEVANT, C. A., HAMMILL, W. E., URAMI, S., and CHOW, S. Flow characteristics of human erythrocytes through polycarbonate sleeves. *Science* 157 825-827 (1967).
- 8 GRIGG, M. E. and GRIGGERS, A. J. Action of some centrally acting drugs on ion transport in red cells. *Science* 123 939-940 (1956).
- 9 HOLMES, D. E. and FRETTE, L. H. Effects of phenothiazine derivatives on biological membranes. Drug-induced changes in electron spin resonance spectra from spin-labeled erythrocyte ghost membranes. *J. Pharmac. exp. Ther.* 173 78-84 (1969).
- 10 JACOB, H. S. Defective membrane microfilaments in the etiology of hereditary spherocytosis; in *Erythrocytes, thrombocytes, leucocytes. Recent advances in membrane and metabolic research. 2nd Int. Symp., Vienna*, pp. 58-61 (Thieme, Stuttgart 1972).

very similar to HS spherocytes as regards morphology osmotic resistance, membrane lipid composition and flexibility In this study formation of spherocyte like red cells of increased fragility was also observed in the presence of high concentrations of vinblastine but the filtration pressure, which was used as a measure of red cell rigidity remained normal It was concluded, therefore, that the vinblastine causes primarily increased red cell fragility Thus the mechanism of the vinblastine effect on red cell membranes would seem to be different from that causing altered rigidity in HS spherocytes.

Several studies on the effect of chlorpromazine on plasma and red cell membranes have been carried out in which effects on membrane ion transport [8 21] red cell morphology [4] membrane protein composition [9] and more recently the adenyl cyclase system [19] have been monitored. A common feature of these studies has been the observation of chlorpromazine induced formation of stomatospherocytes resembling the spherocytes seen in HS patients Our studies verify similarities in morphology and osmotic resistance between HS cells and red cells treated with chlorpromazine in concentrations greater than 50 $\mu\text{mol/l}$ The transport of Na^+ and K^+ is known to be inhibited by chlorpromazine [8] However the increase in osmotic resistance seen with 7 $\mu\text{mol/l}$ would on the other hand agree with reports that small concentrations of chlorpromazine stabilize the red cell membrane [23] The inhibition of adenyl cyclase activity by chlorpromazine reported by OSKES *et al.* [19] is of interest as impairment of membrane protein phosphorylation has been observed in spherocytes from HS patients and also in vinblastine-treated red cells [13 16]

Judged from the filtration experiments the chlorpromazine-treated red cells resembled HS spherocytes more than the vinblastine treated erythrocytes. The previously observed inhibitory effect of chlorpromazine on red cell Ca^{++} transport [21] and the altered Ca^{++} metabolism in HS spherocytes [6] might also reflect a similar red cell membrane protein abnormality Resolution of this problem must await progress towards the understanding of the relationship between the structural components and energy metabolism in the red cell membrane.

Red cell flexibility is considered to be a function of the rigidity and fragility of its membrane. Consequently the method described here was designed to analyse the contribution of these two determinants to changes in the red cell flexibility by estimating alterations of fragility from the degree of hemolysis induced by filtration through polycarbonate membranes and by registering alterations in rigidity from the filtration pressure. The

Folic Acid Binding Protein in Chronic Granulocytic Leukaemia The Effect of Methotrexate

R. COTROCHER, M. L. PACHOR, L. M. BAMBARA, A. AMBROSETTI
and G. DE SANDRE

Istituto di Patologia Medica II (Dir. Prof. G. De Sandre), Università di Padova,
Verona

Key Words. Folate binding protein · Folic acid · Methotrexate

Abstract. Folic acid binding protein (FABP) has been measured in the supernatant of leukocytes of 12 patients affected with chronic granulocytic leukaemia (CGL). The folate binding capacity ranged from 1.37 to 697.52 pg/mg protein, showing considerable heterogeneity. When the supernatant was preincubated with methotrexate (MTX), the inhibition of folic acid binding was complete in some cases whereas in others it was negligible: these findings have been confirmed by studying the ^3H -MTX binding capacity by the same supernatants. In this case the range of bound ^3H -MTX varied from 0.00 to 927 pg/mg protein. The presence of a binder in the cytoplasm of leukocytes might represent a new step in the regulation of endogenous folate metabolism. The MTX, widely used as an antifolate drug, may also be bound by FABP of CGL leukocytes, although in different amounts from case to case: this finding suggests a new point of interference of MTX in the folate metabolism.

It has also been demonstrated that FABP which is present in serum, may reduce the uptake of folate by leukocytes opening a new field of investigation on the megakaryoblastic transformation.

Introduction

The presence of folate binding protein (FABP) was first demonstrated by ROTHENBERG [15] in the cytoplasm of leukocytes of patients with chronic granulocytic leukaemia (CGL). A similar factor has been detected in human milk [19] in the epithelial cells of small intestine in rats [10] in rat liver [3, 4] in human liver and duodenum [4] and in serum of normal subjects and in patients with a variety of diseases [17]. The biological sig-

- 11 JACOB H. S. RUBY A. OVERLAND E. S., and MAZIA, D. Abnormal membrane protein of red blood cells in hereditary spherocytosis. *J. clin. Invest.* 50 1800-1805 (1971).
- 12 JACOB, H. S. AMSEN, T. and WHITE, J. Membrane microfilaments of erythrocytes: alteration in intact cells reproduces the hereditary spherocytosis syndrome. *Proc. natn. Acad. Sci. USA* 69 471-474 (1972).
- 13 JACOB H. S. MATSUMOTO, N. and YAWATA, Y.. Phosphorylation of a filamentous protein (spectrin) and RBC shape. Defect in hereditary spherocytosis (HS). *Clin. Res.* 22 639A (1974).
- 14 LACELLE, P. L. and WEED R. I. Reversibility of abnormal deformability and permeability of the hereditary spherocyte. *Blood* 34 858 (1969).
- 15 MARCHION, V. T. and STEIN, E. Selective solubilization of a protein component of the red cell membrane. *Science* 159 203-204 (1968).
- 16 MATSUMOTO N. YAWATA, Y. and JACOB, H. S. Association of decreased membrane protein phosphorylation with red blood cell spherocytosis. *Blood* 49 233-239 (1977).
- 17 MURPHY J. R. The influence of pH and temperature on some physical properties of normal erythrocytes and erythrocytes from patients with hereditary spherocytosis. *J. Lab. clin. Med.* 69 758-775 (1967).
- 18 ORDRELL, T. Isolation and characterization of an actin-like protein from membranes of human red cells. *Br. J. Haemat.* 35 453-458 (1977).
- 19 OSMO, J. B. CHRISTOFFERSEN T. MÖLLAND, J. and ÖRT, I. Chlorpromazine and hormonal elevation of cyclic AMP content in turkey erythrocytes and in perfused rat heart and liver. *Acta pharmac. tox.* 38 195-208 (1976).
- 20 PAGE, L. and CULVER, R.. A syllabus of laboratory examination in clinical diagnosis, p. 116 (Harvard University Press, Cambridge 1960).
- 21 SCHATZMANN H. J., in GUTHBERT Symposium on calcium and cellular function, pp. 85-95 (MacMillan, New York 1970).
- 22 SCHMID-SCHÖNBEIN H. WEISS, J. and LUDWIG H.. A simple method for measuring red cell deformability in models of the microcirculation. *Blut* 26 369-379 (1973).
- 23 SEIDMAN P. and WEINSTEIN J.. Erythrocyte membrane stabilization by tranquilizers and antihistamines. *Biochem. Pharmacol.* 15 1737-1752 (1966).
- 24 TITTEL, P. Le test de la filtrabilité érythrocytaire (TFE). Une méthode simple d'étude de certaines propriétés microhéologiques des globules rouges. *Nouv. Revues fr. Hémat.* 7 195-214 (1967).

by ultrafiltration (Amicon System) to 5 ml (protein concentration 10 mg/ml), and eluted at 4°C from Sephadex G-200 column (80 × 2.5 cm) equilibrated with 0.05 M sodium citrate buffer pH 8 and calibrated with blue dextran, hemoglobin, and folic acid. 150 fractions were collected and fractions from 45 to 60 - which contain the binder as previously demonstrated (fig. 1) - were pooled and concentrated by ultrafiltration to 1 mg/ml protein. The binding capacity of the partially purified binder was checked using the small column system described above.

Uptake of ³H-PteGlu by CGL Leukocytes

0.5 ml of saline/plasma (v/v) suspension of CGL leukocytes (10^7 cells) were incubated at 37°C with 5.9 ng of ³H-PteGlu for 0, 15, 30, 60, 120, and 180 min. The samples were then washed three times with 50 vol of phosphate-buffered saline to remove the residual radioactivity. The same procedure was followed incubating samples of CGL leukocytes with ³H-PteGlu in the presence of 200 µg of partially purified binder. The samples were then lysed and the supernatant prepared as described above. The radioactivity of 0.5 ml of each supernatant was measured in Instagel.

Protein Estimation

The amount of protein was measured either at 280 nm or with the Lowry method [11].

Results

The clinical data on the patients studied are given in table I. Figure 2 shows the chromatographic pattern of radioactivity after elution of ³H-PteGlu previously incubated with supernatant of leukocytes from CGL or normal subjects. The first peak represents the bound folate whereas the second represents free folate. It is possible to calculate the percentage and therefore the picograms of bound folate per milligram of protein in the original supernatant. In our cases of CGL, the binding capacity ranged widely from 1.37 to 698 pg/mg protein (table II). When the supernatants were preincubated with cold MTX, the amount of bound ³H-PteGlu again varied from case to case (table II). In some cases the inhibition was almost complete (P. M., Z. L.) while in others the inhibition by cold MTX was minimal or absent (P. S., R. M., F. V.). The binding capacity of 2 of the patients (P. M., and P. S.) illustrates this variability: the amount of ³H-PteGlu bound by both the supernatants was very high when the supernatants were preincubated with cold MTX the inhibition of ³H-PteGlu binding was complete in 1 case (P. M.) but virtually absent in the other (P. S.) (fig. 3).

nificance of these proteins still remains unknown. The aim of the present study was to determine the effect of methotrexate (MTX) on the binding of folate by FABP and the effect of FABP on the uptake of radioactive folic acid (^3H PteGlu) by leukocytes.

Material and Methods

Patients

Leukocytes were prepared from venous blood of 12 patients (7 males and 5 females) with CGL. All the patients were Philadelphia positive and untreated at the time of the present studies (table I). The leukocytes were collected after sedimentation of heparinized blood (2 h at room temperature) and washed three times with cold phosphate-buffered saline as previously described [5].

Reagents

Radioactive folic acid (^3H PteGlu) of a specific activity of 37 Ci/mmol and labelled MTX (specific activity 6 Ci/mmol) were supplied by The Radiochemical Center Amersham. The cold MTX was purchased from Sigma.

Preparation of the Supernatant

The leukocytes were lysed after freezing and thawing in 0.05 M sodium citrate buffer pH 8, as suggested by ROTUNDO [15] and centrifuged at 40,000 g for 180 min. The supernatants were then stored at -20°C until used.

Labelling of the Supernatant

Aliquots of 0.2 ml of each supernatant were mixed with 0.3 ml of sodium citrate buffer and incubated with 20 μl (5.9 ng) of ^3H PteGlu for 15 min at 37°C (Control). To study the competition of MTX for the folate binding, the supernatant was incubated for 15 min at 37°C with cold MTX (1 mg in 20 μl) before adding ^3H -PteGlu, suitable controls were set up preincubating the supernatant with 20 μl of distilled water instead of cold MTX. The capacity of the supernatant to bind directly ^3H MTX was also determined incubating an equal aliquot of supernatant with 20 μl (6 ng) of ^3H MTX.

Chromatographic Studies

0.5 ml of labelled supernatant (Control) were eluted from Sephadex G-75 column (13 \times 0.6 cm) equilibrated with 0.05 M sodium citrate buffer pH 8 at 4°C . 35 of 0.5-ml fractions were collected in vials filled with 40 ml of Instagel (Packard). The radioactivity of each fraction was measured in a Liquid Scintillation Counter (Packard) and plotted against fraction number. The percentage of bound folate was determined integrating the area of the radioactive peak. The same technique was followed eluting supernatant labelled with ^3H PteGlu preincubated with cold MTX or labelled with ^3H -MTX.

The binder used in the uptake studies (see below) was prepared from leukocytes of one of the CGL-Ph⁺-positive patients (P.M.). The supernatant was concentrated

by ultrafiltration (Amicon System) to 5 ml (protein concentration 10 mg/ml), and eluted at 4 °C from a Sephadex G-200 column (80 × 2.5 cm) equilibrated with 0.05 M sodium citrate buffer pH 8 and calibrated with blue dextran, hemoglobin, and folic acid. 150 fractions were collected and fractions from 45 to 60 – which contain the binder as previously demonstrated (fig. 1) – were pooled and concentrated by ultrafiltration to 1 mg/ml protein. The binding capacity of the partially purified binder was checked using the small column system described above.

Uptake of ³H-PteGlu by CGL Leukocytes

0.5 ml of a saline/plasma (v/v) suspension of CGL leukocytes (10⁶ cells) were incubated at 37 °C with 5.9 ng of ³H-PteGlu for 0, 15, 30, 60, 120, and 180 min. The samples were then washed three times with 50 vol of phosphate-buffered saline to remove the residual radioactivity. The same procedure was followed incubating samples of CGL leukocytes with ³H-PteGlu in the presence of 200 µg of partially purified binder. The samples were then lysed and the supernatant prepared as described above. The radioactivity of 0.5 ml of each supernatant was measured in Instagel.

Protein Estimation

The amount of protein was measured either at 280 nm or with the Lowry method [11].

Results

The clinical data on the patients studied are given in table I. Figure 2 shows the chromatographic pattern of radioactivity after elution of ³H-PteGlu previously incubated with supernatant of leukocytes from CGL or normal subjects. The first peak represents the bound folate whereas the second represents free folate. It is possible to calculate the percentage and therefore the picograms of bound folate per milligram of protein in the original supernatant. In our cases of CGL, the binding capacity ranged widely from 1.37 to 698 pg/mg protein (table II). When the supernatants were preincubated with cold MTX, the amount of bound ³H-PteGlu again varied from case to case (table II). In some cases the inhibition was almost complete (P. M., Z. L.) while in others the inhibition by cold MTX was minimal or absent (P. S., R. M., F. V.). The binding capacity of 2 of the patients (P. M., and P. S.) illustrates this variability: the amount of ³H-PteGlu bound by both the supernatants was very high when the supernatants were preincubated with cold MTX the inhibition of ³H-PteGlu binding was complete in 1 case (P. M.) but virtually absent in the other (P. S.) (fig. 3).

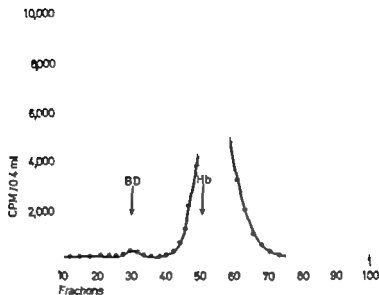


Fig 1 Chromatographic profile of ^3H PteGlu incubated with supernatant from leukocytes of P M BD = Blue dextran Hb = hemoglobin — = ^3H PteGlu.

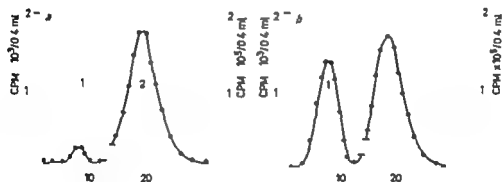


Fig 2 Chromatographic profile of ^3H PteGlu incubated with supernatants from normal (a) and leukaemic leukocytes (b). The first peak represents the bound folate, the second the free folate.

The elution studies using ^3H MTA labelled supernatants confirm the heterogeneity of the binding the picograms of ^3H MTA bound per milligram of protein ranged from 0.00 to 927 (table II). Figure 4 shows the uptake of ^3H PteGlu by CGL leukocytes. The influx of the vitamin, rapid during the first 30 min, increased progressively until the end of the experiment. When the uptake study was performed in the presence of FABP the incorporation of ^3H PteGlu into CGL leukocytes was signifi-

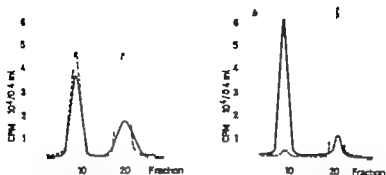


Fig 3. Elution patterns of ^3H PteGlu incubated with supernatants from leukocytes of 2 patients (a = P.S., b = P.M.) (—) the same experiment repeated preincubating supernatant with cold MTX (---).

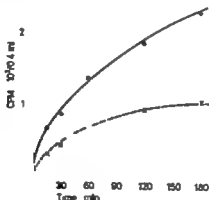


Fig 4. Uptake of ^3H PteGlu by CGL leukocytes without (—) or with (---) FABP in the medium.

cantly reduced being at the end of the experiment about 50% less than that in the control.

Discussion

The mechanism of action of MTX has been attributed to the blocking of tetrahydrofolate synthesis from dihydrofolate which causes the depletion of folate coenzymes slowing the folate-dependent reactions such as

Table I Some clinical data of CGL leukaemic patients

No.	Name	Sex	Age	Hb g/dl	WBC/pl	Differential count of white cells in peripheral blood, %						
						PMN	MM	MC	PMC	MBL	BL	L
1	M. F	F	53	9.4	87,100	64	20	6	13	4	7	4
2	P. M.	M	24	7.6	89,300	54	20	12	8	4	-	2
3	P. S.	M	18	11.6	155,000	50	15	16	10	1	4	4
4	T. A.	M	38	8.6	138,000	66	6	12	10	3	-	3
5	R. M.	M	52	11.5	49,000	85	4	4	-	-	-	7
6	S. P.	F	41	8.4	52,300	42	13	10	-	18	17	2
7	C. G.	F	33	19.0	85,000	54	9	9	7	4	12	5
8	F. V.	M	47	8.4	160,000	65	10	2	3	-	-	20
9	Z. L.	M	42	9.5	104,000	27	-	-	-	-	24	49
10	P. V.	F	52	7.6	132,000	72	10	9	2	-	6	2
11	D. F.	M	21	13.5	116,000	63	11	15	3	2	-	6
12	S. G.	F	47	9.5	350,000	28	2	10	25	15	8	12

WBC = White blood cells PMN = polymorphonuclear neutrophils MM = meta-myelocytes MC = myelocytes PMC = promyelocytes MBL = myeloblasts BL = undifferentiated blasts L = lymphocytes.

Table II Different binding capacity of CGL leukocytes effect of MTX

CGL patients		pg ³ H-PteGlu/mg protein		Inhibition, %	pg ³ H MTX/mg protein
no.	name	without MTX	with MTX		
1	M. F.	18.17	15.61	14.1	n.t.
2	P. M.	697.52	2.17	98.7	927.21
3	P. S.	457.52	441.04	0.8	14.25
4	T. A.	4.76	4.42	7.2	0.06
5	R. M.	4.41	4.50	0.0	0.00
6	S. P.	1.37	0.40	70.8	2.25
7	C. G.	11.74	11.70	0.4	0.86
8	F. V.	82.89	83.05	0.0	1.09
9	Z. L.	189.77	0.05	95.6	213.74
10	P. V.	13.41	10.01	25.5	4.83
11	D. F.	35.44	30.68	13.5	7.21
12	S. G.	9.41	6.91	23.6	n.t.

n. t. = Not tested.

the synthesis of oxythymidilate from deoxyuridilate [1] This result is determined by the binding MTX with the dihydrofolate-reductase. The effectiveness of MTX therapy depends, as suggested by GOLDMAN [8], on several steps: (a) the transfer of MTX through the membrane (b) the unsaturated dihydrofolate reductase binding sites (c) the level of the tetra hydrofolate-cofactor stores, (d) the activity of enzyme pathways necessary for the interconversion of folate coenzymes (e) the existence of alternative metabolic pathways which might circumvent MTX-induced metabolic block [18] (f) the percentage of cell population in S-phase and the growth rate of the entire tumor population, (g) the exposition of the cell to the circulating MTX, (h) the rate of synthesis of new dihydrofolate-reductase (i) the level of intracellular-free MTX which maintains the folate-reductase in a saturated state and blocks the new synthesized folate-reductase.

As it appears from the above considerations the intracellular-free MTX plays a crucial role in the folate metabolism and the effectiveness of therapy. Therefore, the presence of a binder in the cytosol of some CGL which binds MTX is of great importance. Our results show that not all the CGL supernatants bound MTX, the MTX binding capacity in the CGL leukocyte supernatants ranged from 927.21 to 0 pg/mg protein, this finding may be explained either by the presence of more than one type of binders - of similar molecular weight - with different affinity for MTX, the proportion of which may vary from case to case, or by different saturation of the binder by the endogenous folates. Moreover it is possible to postulate a modification of the structure of binder induced by the neoplastic transformation, which causes a different affinity for the drug.

The significance of the new binder remains still poorly understood its role in the effectiveness of therapy with MTX is unknown. One might hypothesize that the binder subtracts the drug to its natural target (i.e. dihydrofolate-reductase) preserving the cell from the toxic effect of MTX. On the contrary it might store the drug prolonging its action against the cell. Certainly it will interfere with the intracellular pool of the folate or MTX and with their movement in and out of the cell.

The binder has been demonstrated in serum of patients with different kinds of diseases [2, 6, 7, 9, 12-14, 16, 22] our results show that FABP will reduce the uptake of folate by the leukemic cells (and probably of MTX) as demonstrated also by WAXMAN and SCHREIBER [20] This effect should be kept in mind when the patients have to be treated either with folate or with MTX.

Table I Some clinical data of CGL leukaemic patients

No.	Name	Sex	Age	Hb g/dl	WBC/ μ l	Differential count of white cells in peripheral blood, %						
						PMN	MM	MC	PMC	MBL	BL	L
1	M. F	F	53	9.4	87,100	64	20	6	13	4	7	4
2	P. M.	M	24	7.6	89,300	54	20	12	8	4	-	2
3	P. S.	M	18	11.6	155,000	50	15	16	10	1	4	4
4	T. A.	M	38	8.6	133,000	66	6	12	10	3	-	3
5	R. M.	M	52	11.5	49,000	85	4	4	-	-	-	7
6	S. P.	F	41	8.4	52,300	42	13	10	-	16	17	2
7	C. G.	F	33	19.0	85,000	54	9	9	7	4	13	5
8	F. V.	M	47	8.4	160,000	65	10	2	3	-	-	20
9	Z. L.	M	42	9.5	104,000	27	-	-	-	-	24	49
10	P. V.	F	52	7.6	132,000	72	10	9	2	-	6	2
11	D. F.	M	21	13.5	116,000	63	11	15	3	2	-	6
12	S. G.	F	47	9.5	350,000	28	2	10	23	15	8	12

WBC = White blood cells PMN = polymorphonuclear neutrophils MM = meta-myelocytes MC = myelocytes PMC = promyelocytes MBL = myeloblasts BL = undifferentiated blasts L = lymphocytes.

Table II Different binding capacity of CGL leukocytes effect of MTX

CGL patients		pg ³ H PteGln/mg protein		Inhibition, %	pg ³ H-MTX/mg protein
no.	name	without MTX	with MTX		
1	M. F.	18.17	15.61	14.1	n.t.
2	P. M.	697.52	2.17	98.7	927.21
3	P. S.	457.52	441.04	0.8	14.25
4	T. A.	4.76	4.42	7.2	0.06
5	R. M.	4.41	4.50	0.0	0.00
6	S. P.	1.57	0.40	70.8	2.25
7	C. G.	11.74	11.70	0.4	0.86
8	F. V.	82.89	83.05	0.0	1.09
9	Z. L.	189.77	0.05	95.6	213.74
10	P. V.	13.41	10.01	25.5	4.83
11	D. F.	35.44	30.68	13.5	7.21
12	S. G.	9.41	6.91	23.6	n.t.

n.t. = Not tested.

- 19 WAXMAN, S. and SCHREINER, C.. Characteristics of folic acid binding protein in folate-deficient serum. *Blood* 42, 291-301 (1973).
- 20 WAXMAN, S. and SCHREINER, C.. The role of folic acid binding protein (FABP) in the cellular uptake of folate. *Proc. Soc. exp. Biol. Med.* 147 760-764 (1974).
- 21 ZAMBEROWSKI, M. and WAGNER, C.. High molecular weight complexes of folic acid in mammalian tissues. *Biochem. biophys. Res. Commun.* 60 81-87 (1974).
- 22 ZETZNER, A. and DOLY F. H.. New evidence for a binding principle specific for folates as a normal constituent of human serum. *Clin. Chem.* 20 1313-1319 (1974).

References

- 1 BERTINO, J. R. The mechanism of action of the folate antagonists in man. *Cancer Res.* 23 1286 (1963)
- 2 COLEMAN, N. and HERBERT V.. Evidence for granulocyte related and liver related binders in human serum and renal glomerular filtration of folate binder. *Clin. Res.* 22 700 (1974)
- 3 CORROCHIER, R., DE SANDRE, G., PACHOR, M. L., and HOFFRAND A. V.. Hepatic protein binding of folate. *Clin. Sci. mol. Med.* 46 331-334 (1974)
- 4 CORROCHIER, R., PACHOR, M. L., BAMBARA, L. M., PERONI, A., and DE SANDRE, G. Folate binding proteins in human digestive system. *Rend. Gastroent.* 8 29-32 (1976)
- 5 CORROCHIER, R., BAMBARA, L. M., PACHOR, M. L., PIZZOLO, G., PERONI, A. and DE SANDRE, G. Folate binding protein in myeloproliferative disorders. *Haematologica* 61 269-277 (1976).
- 6 DA COSTA, M. and ROTHENBERG S. P.. Appearance of a folate binder in leukocytes and serum of women who are pregnant or taking oral contraceptives. *J. Lab. clin. Med.* 83 207-214 (1974)
- 7 EICHNER, E. R., PAINE, C. J., DICKSON, V. L., and HARGROVE, M. D. Clinical and laboratory observations on serum folate-binding protein. *Blood* 46 599-609 (1975).
- 8 GOLDMAN D. Analysis of the cytotoxic determinants of methotrexate (NSC 740) a role for free intracellular drug. *Cancer Chem.* 6 51-61 (1975).
- 9 HINEA, J. D., KAMEN, B., and CASTON, H. Abnormal folate bindings in azotemic patients. *Blood* 42 997 (1973)
- 10 LESLIE, G. I. and ROWE, F. B. Folate binding by the brush border membrane proteins of small intestinal epithelial cells. *Biochemistry* 11 1696 (1972)
- 11 LOWRY O. H., ROSEBROUGH N. J., FARR, A. L., and RANDALL, R. J. Protein measurement with the Folin-phenol reagent. *J. biol. Chem.* 193 265-275 (1951).
- 12 MARKKANEN T. Pteroylglutamic acid (PGA) activity of serum in gel filtration. *Life Sci.* 7 887-895 (1968).
- 13 MARKKANEN, T. and PILTOLA, O. Carrier proteins of folic acid activity in human serum. *Acta haemat.* 45 175-179 (1971).
- 14 MARKKANEN, T., PAJULA, R. L., HIMANEN, P. and VIRTANEN S.. Serum folic acid activity (*L. casei*) in Sephadex gel chromatography. *J. clin. Path.* 26 486-493 (1973).
- 15 ROTHENBERG, S. P.. A macromolecular factor in some leukemic cells which binds folic acid. *Proc. Soc. exp. Biol. Med.* 133 428-432 (1970).
- 16 ROTHENBERG, S. P. Application of competitive ligand binding for the radioassay of vitamin B₁₂ and folic acid. *Metabolism* 22 1075-1082 (1973)
- 17 ROTHENBERG, S. P. and DA COSTA, M. Folate binding proteins and radioassay for folate. *Clin. Haemat.* October 1976.
- 18 Sneider T. W. and POTTER, V. R.. Alternative de novo and salvage pathways to thymidine triphosphate synthesis: possible implication for cancer chemotherapy. *Cancer Res.* 29 2389 (1969).

of African slaves or secondary dispersions of the African mutation by mixed populations of the Arabian peninsula. An alternative hypothesis, relating anthropologic affinities of Greek, Etr-Turk, and Indian Vedddot populations with hemoglobin S, proposes that the original sickle cell hemoglobin mutation occurred in Arabia and spread west to Africa and north to Europe [22]

Of specific interest are reports of hemoglobin S occurring in white persons with no African ancestry [4-17], reviving as an alternative to the single mutation theory the possibility that sickle cell hemoglobin arose as multiple mutations in different geographic areas [32]. A review of the case reports of hemoglobin S in white individuals reveals that the majority are descendants of Sicilian emigrants [7-10, 18-20, 24-26, 29-36] and, therefore, an assessment of African admixture in Sicilian populations is fundamental in evaluating the origin of hemoglobin S in these individuals. There is abundant historical evidence linking indigenous Sicilian and African populations. Thus, it is not surprising that reports of hemoglobin S in native Sicilians were among the first in nonblack populations [13-35]. However direct genetic evidence of African admixture in Sicilian populations has not been published.

The present report describes a survey of hemoglobin phenotypes and blood group markers of anthropologic significance in native Sicilians as an approach to determining the origin of sickle cell hemoglobin in white persons of Sicilian ancestry.

Materials and Methods

Study Populations

Blood specimens were obtained from two groups of native Sicilians. One group consisted of 64 individuals from 21 families in which at least one family member was known to be carrier of hemoglobin S. 43 subjects were inhabitants of the southern provinces of Catania and Syracuse on the Ionian coast and 21 were inhabitants of the northern Tyrrhenian coastal region of Palermo. The family group was selected to approximate the genetic counterpart of Sicilian emigrants with hemoglobin S.

The second group consisted of 100 unrelated native Sicilians who lived in the same provinces as the families. The group of unrelated Sicilians was selected to evaluate the approximate frequencies of the specific African markers in the regional populations that are not necessarily affected by hemoglobin S.

Hemoglobin Phenotypes

Individual diagnoses of sickle cell trait (hemoglobin AS) and sickle cell disease (hemoglobin SS) were based on the percentages of hemoglobin A and hemoglobin S

Blood Group Phenotypes and the Origin of Sickle Cell Hemoglobin in Sicilians¹

S GERALD SANDLER, GINO SCHILIRÒ, ANTONIO RUSSO,
SALVATORE MUSUMECI and ELIEZER A. RACHMILEWITZ

Blood Bank and Department of Hematology
Hadassah-Hebrew University Medical Center Jerusalem, and
Clinica Pediatrica, Policlinico dell'Università di Catania, Catania

Key Words Hemoglobin S Blood groups Sicily

Abstract. As an approach to investigating the origin of sickle cell hemoglobin (hemoglobin S) in white persons of Sicilian ancestry two groups of native Sicilians were tested for blood group evidence of African admixture. Among 100 unrelated Sicilians, the phenotypes cDe(Rh₀) and Py(a-b-) and the antigens V(hrv) and Ia₂, which are considered to be African genetic markers, were detected in 11 individuals. Among 64 individuals from 21 families with at least one known hemoglobin S carrier African blood group markers were detected in 7 (11%). These findings indicate that hemoglobin S is only one of multiple African genes present in contemporary Sicilian populations. The occurrence of hemoglobin S in white persons of Sicilian ancestry is considered to be a manifestation of the continuing dissemination of the original African mutation.

The present distribution of sickle cell hemoglobin (hemoglobin S) in nonblack populations of Europe, Asia, Australia, North African and the Americas is considered to reflect the dissemination of a single Equatorial African mutation by historical population migrations [14 15 17 23 31]. Accordingly foci of hemoglobin S in the Mediterranean littoral are regarded to be either direct admixtures resulting from historical importation

¹ This study was supported in part by the Morris Lewinsohn Fund for Hematology Research (SGS) and by a grant from the National Foundation of March of Dimes (E. A. R.). E. A. R. is an established investigator of the Chief Scientist Bureau, The Israel Ministry of Health.

Table I. Results of hematologic tests (mean values) and hemoglobin phenotypes for Sicilians from families with sickle cell hemoglobin

Hemoglobin genotype	No. of individuals	Hb g/dl	RBC 10^6	MCHC g/dl	MCH pg	Hemoglobin electrophoresis		
						Aa	F %	S
Hb AA	4	14.1	5.0	30.5	26.5	2.2	1.0	0
Hb AS	1	12.3	4.5	30.0	27.0	2.3	1.3	40.0
Hb SS	5	7.9	3.3	26.8	25.4	1.8	9.2	82.0
Hb A/ β -thal	11	13.0	5.0	29.1	16.0	5.3	1.5	0
Hb S/ β -thal	11	10.7	4.2	28.2	25.2	2.0	11.3	79.8

was detected in 1 additional person (table II). S-s- and G^a were not detected. Overall, 7 of the 64 family members (11%) had phenotypic evidence of African admixture. An additional 4 persons from the family groups with the phenotype CcDee(Rh,rh) were identified to be genotypically R¹R by analysis of family pedigrees. Among the 100 unrelated Sicilians, cDe(Rh₀), V(hr⁺), Fy(a-b-) and J^s were detected individually in 12 persons (12%) S-s- and G^a were not detected in the group of unrelated persons with hemoglobin AA.

Discussion

There is both historical and genetic evidence favoring an African origin of the sickle cell hemoglobin gene in Sicilian populations.

The historical evidence dates from the importation of African slaves during the Phoenician era, followed by subsequent invasions of Sicily by Carthaginians and Tunisian Berbers [12, 34]. North African Saracens and Sudanese soldiers entered Sicily during Islamic conquests of the 8th and 9th centuries [34]. As a consequence of these and lesser historical events, an estimated 10% of contemporary Sicilians are thought to carry the sickle cell trait [5].

The genetic evidence favoring an African origin for the hemoglobin S gene in Sicilian populations is derived from the association of hemoglobin S with specific African genetic markers, namely cDe(Rh₀), V(hr⁺), Fy(a-b-) and J^s suggesting that hemoglobin S is only one, albeit the most apparent, of multiple African genes in the Sicilian admixture. In certain other situations where 'parent' white and black populations are clearly defin-

by cellulose acetate electrophoresis, as previously described [11]. Diagnoses of β thalassemia trait (hemoglobin A/ β thal) were based upon characteristic erythrocyte morphology, elevated hemoglobin A₂ levels, decreased osmotic fragility and confirmatory family pedigrees. The differentiation between sickle cell trait β thalassemia (hemoglobin S/ β -thal) and Hb SS, which in some cases was not possible by laboratory studies alone, was based on analysis of the family pedigrees.

Blood Group Markers

Blood group tests were performed by standard tube methods for the following antigens: A, A₁, B, D(Rh₀), D^u(Rh₀), C(rh'), E(rh''), c(rh'), e(rh''), V(hrv), Go^a, M, N, S, s, Fy^a, Fy^b, Jk⁻, k, K and Jk^a. Except for anti V(hrv) which had been generously provided by Mrs. CHARLA H. LESTER, Paul L. Hoxworth Blood Center, Cincinnati, Ohio, all antisera were commercial reagents used in accordance with manufacturer's recommendations (Gamma Biologicals, Houston, Tex.). The phenotypes cDe(Rh₀), Fy(a-b-) and S-s- and the antigens V(hrv), Jk^a and Go^a were selected as genetic markers of African origin on the basis of evidence which is summarized in detail by MOURANT *et al.* [27], RACE and SANGER [30] and BOYD [6]. The presence of cDe(Rh₀), Fy(a-b-) V(hrv) or Jk^a is considered to be virtually diagnostic of African origin and combination of these markers without evidence of African ancestry has not been reported. Go^a and S-s- are no less specific [30, 33] but because of the relatively lower frequencies in indigenous and mixed African populations, these markers are generally less useful for anthropologic distinctions. The Duffy amorph Fy(a-b-) has been identified to be the product of the Fyⁱ gene (FyⁱFyⁱ or FyⁱFy^j) [3], which is probably the specific African marker. However, since the present tests were performed only with anti-Fy^a and anti-Fy^b, the Fy(a-b-) designation is preserved. Because of the relatively small size and selectivity of the population samples, calculations of gene frequencies were not performed.

Results

Hemoglobin Phenotypes

Sickle cell hemoglobin was detected in 49 of the 64 family members (77%), including 5 homozygotes (hemoglobin SS), 21 heterozygotes (hemoglobin AS), and 23 heterozygotes with β thalassemia (hemoglobin S/ β thal) (table 1). An additional 11 family members were diagnosed as having the β -thalassemia trait (hemoglobin A/ β -thal) and 4 were hematologically and electrophoretically normal (hemoglobin AA). Hb S was not detected among the Sicilians who all had normal adult hemoglobin (hemoglobin AA).

Blood Group Markers

In the family group, cDe (Rh₀), V(hrv), Fy(a-b-) and Jk^a were detected individually in 6 persons and the combination cDe(Rh₀) and V(hrv)

Table I. Results of hematologic tests (mean values) and hemoglobin phenotypes for Sicilians from families with sickle cell hemoglobin

Hemoglobin genotype	No. of individuals	Hb g/dl	RBC 10^6	MCHC g/dl	MCH %	Hemoglobin electrophoresis		
						A ₂	F %	S
Hb AA	4	14.1	5.0	30.5	26.5	2.1	1.0	0
Hb AS	21	12.3	4.5	30.0	27.0	2.3	1.3	40.0
Hb SS	5	7.9	3.3	26.8	25.4	1.8	9.2	82.0
Hb A/ β -thal	11	13.0	5.0	29.1	16.0	5.3	1.5	0
Hb S/ β -thal	23	10.7	4.2	28.2	25.2	2.0	11.3	79.8

was detected in 1 additional person (table II). S-s- and G^o were not detected. Overall, 7 of the 64 family members (11%) had phenotypic evidence of African admixture. An additional 4 persons from the family groups with the phenotype CcDee(Rh,rh) were identified to be genotypically R R by analysis of family pedigrees. Among the 100 unrelated Sicilians, cDe(Rh₀), V(hr⁺), Fy(a-b-) and J^s were detected individually in 12 persons (12%) S-s- and G^o were not detected in the group of unrelated persons with hemoglobin AA.

Discussion

There is both historical and genetic evidence favoring an African origin of the sickle cell hemoglobin gene in Sicilian populations.

The historical evidence dates from the importation of African slaves during the Phoenician era, followed by subsequent invasions of Sicily by Carthaginians and Tunisian Berbers [12, 34]. North African Saracens and Sudanese soldiers entered Sicily during Islamic conquests of the 8th and 9th centuries [34]. As a consequence of these and lesser historical events, an estimated 1% of contemporary Sicilians are thought to carry the sickle cell trait [5].

The genetic evidence favoring an African origin for the hemoglobin S gene in Sicilian populations is derived from the association of hemoglobin S with specific African genetic markers, namely cDe(Rh₀), V(hr⁺), Fy(a-b-) and J^s, suggesting that hemoglobin S is only one, albeit the most apparent, of multiple African genes in the Sicilian admixture. In certain other situations where "parent" white and black populations are clearly defin-

by cellulose acetate electrophoresis, as previously described [11]. Diagnoses of β -thalassaemia trait (hemoglobin A/ β -thal) were based upon characteristic erythrocyte morphology, elevated hemoglobin A₂ levels, decreased osmotic fragility and confirmatory family pedigrees. The differentiation between sickle cell trait β -thalassaemia (hemoglobin S/ β -thal) and Hb SS, which in some cases was not possible by laboratory studies alone, was based on analysis of the family pedigrees.

Blood Group Markers

Blood group tests were performed by standard tube methods for the following antigens A, A₂, B, D(Rh₀), D^u(Rh₀), C(rh'), E(rh''), c(rh'), e(rh''), V(hr'), Go^a, M, N, S, s, Fy^a, Fy^b, Jk^a, Jk^b, k and Js^a. Except for anti-V(hr') which had been generously provided by Mrs. CHARLA H. LEWITT, Paul L. Hoxworth Blood Center, Cincinnati, Ohio, all antisera were commercial reagents used in accordance with manufacturer's recommendations (Gamma Biologicals, Houston, Tex.). The phenotypes cDe(Rh₀), Fy(a-b-), and S-s-, and the antigens V(hr'), Js^a and Go^a were selected as genetic markers of African origin on the basis of evidence which is summarized in detail by MOURANT *et al* [27], RACE and SANDER [30] and BOYD [6]. The presence of cDe(Rh₀), Fy(a-b-), V(hr') or Js^a is considered to be virtually diagnostic of African origin and combination of these markers without evidence of African ancestry has not been reported. Go^a and S-s- are no less specific [30, 33] but because of the relatively lower frequencies in indigenous and mixed African populations, these markers are generally less useful for anthropologic distinctions. The Duffy amorph Fy(a-b-) has been identified to be the product of the *Fyⁱ* gene (*FyⁱFyⁱ* or *FyⁱFy^j*) [3], which is probably the specific African marker. However, since the present tests were performed only with anti-Fy^a and anti-Fy^b, the Fy(a-b-) designation is preserved. Because of the relatively small size and selectivity of the population samples, calculations of gene frequencies were not performed.

Results

Hemoglobin Phenotypes

Sickle cell hemoglobin was detected in 49 of the 64 family members (77%) including 5 homozygotes (hemoglobin SS), 21 heterozygotes (hemoglobin AS) and 23 heterozygotes with β -thalassaemia (hemoglobin S/ β -thal) (table I). An additional 11 family members were diagnosed as having the β -thalassaemia trait (hemoglobin A/ β -thal) and 4 were hematologically and electrophoretically normal (hemoglobin AA). Hb S was not detected among the Sicilians who all had normal adult hemoglobin (hemoglobin AA).

Blood Group Markers

In the family group cDe (Rh₀), V(hr'), Fy(a-b-) and Js^a were detected individually in 6 persons and the combination cDe(Rh₀) and V(hr')

Table I Results of hematologic tests (mean values) and hemoglobin phenotypes for Sicilians from families with sickle cell hemoglobin

Hemoglobin genotype	No. of Individuals	Hb g/dl	RBC 10^6	MCHC g/dl	MCH pg	Hemoglobin electrophoresis		
						A	F %	S
Hb AA	4	14.1	5.0	30.5	26.5	2.2	1.0	0
Hb AS	21	12.3	4.5	30.0	27.0	2.3	1.3	40.0
Hb SS	5	7.9	3.3	26.8	25.4	1.8	9.2	82.0
Hb A/ β -thal	11	13.0	5.0	29.1	16.0	5.3	1.5	0
Hb S β -thal	23	10.7	4.2	28.2	25.2	2.0	11.3	79.8

was detected in 1 additional person (table II). S-s- and Go^a were not detected. Overall, 7 of the 64 family members (11%) had phenotypic evidence of African admixture. An additional 4 persons from the family groups with the phenotype CeDee(Rh₀,rh) were identified to be genotypically R R^a by analysis of family pedigrees. Among the 100 unrelated Sicilians, cDe(Rh₀), V(hr⁺), Fy(a-b-) and Js^a were detected individually in 12 persons (12%). S-s- and Go^a were not detected in the group of unrelated persons with hemoglobin AA.

Discussion

There is both historical and genetic evidence favoring an African origin of the sickle cell hemoglobin gene in Sicilian populations.

The historical evidence dates from the importation of African slaves during the Phoenician era, followed by subsequent invasions of Sicily by Carthaginians and Tunisian Berbers [12, 34]. North African Saracens and Sudanese soldiers entered Sicily during Islamic conquests of the 8th and 9th centuries [34]. As a consequence of these and lesser historical events, an estimated 1% of contemporary Sicilians are thought to carry the sickle cell trait [5].

The genetic evidence favoring an African origin for the hemoglobin S gene in Sicilian populations is derived from the association of hemoglobin S with specific African genetic markers, namely cDe(Rh₀), V(hr⁺), Fy(a-b-) and Js^a suggesting that hemoglobin S is only one, albeit the most apparent, of multiple African genes in the Sicilian admixture. In certain other situations where "parent" white and black populations are clearly defin-

Table II Blood groups of Sicilian families with sickle cell hemoglobin (Hb S) and of unrelated Sicilians with normal adult hemoglobin (Hb AA)

Phenotypes	Individuals from families with known Hb S					Unrelated native Sicilians Hb AA (n = 100)
	Hb AA (n = 4)	Hb AS (n = 21)	Hb SS (n = 5)	Hb A/ β thal (n = 11)	Hb S/ β -thal (n = 23)	
<i>ABO system</i>						
A ₁	2	4	1	4	4	29
A ₂	—	—	2	1	1	11
B	—	3	1	2	3	10
A ₁ B	—	—	—	—	—	6
A ₂ B	—	—	—	—	—	3
O	2	14	1	4	14	41
<i>Rhesus system</i>						
CCDee	2	6	—	2	5	21
CCDEe	—	—	—	—	—	3
CcDEe	2	4	—	2	5	16
CcDEeV	—	—	—	—	—	1
CcDee	—	7	4	3	8	32
CcDeeV	—	1	—	—	—	5
ccdee	—	—	—	—	—	2
ccDEE	—	1	—	1	—	5
ccDee	—	1	—	1	—	—
ccDEe	—	—	1	—	4	4
ccDeeV	—	—	—	1	—	—
ccDEeV	—	—	—	—	—	1
ccdee	—	1	—	1	1	7
<i>Duffy system</i>						
Fy(a+b-)	1	2	3	1	4	—1
Fy(a+b+)	2	11	2	6	15	43
Fy(a-b+)	—	8	—	4	4	36
Fy(a-b-)	1	—	—	—	—	1
<i>Kell system</i>						
Kk Js(a-)	—	4	—	1	3	7
kk Js(a-)	2	17	5	9	19	90
kk Js(a+)	—	—	—	1	1	2
<i>MN system</i>						
MMSS	—	4	1	—	1	10
MMSs	2	2	1	3	5	23
MMss	1	—	—	2	1	12
MNSS	1	—	—	—	—	3

Table II (continued)

Phenotypes	Individuals from families with known Hb S					Unrelated native Sicilians Hb AA (n = 100)
	Hb	Hb	Hb	Hb	Hb	
	AA (n = 4)	AS (n = 1)	SS (n = 5)	A/ β -thal (n = 11)	S/ β -thal (n = 23)	
<i>AfV system</i>						
MN _S a		1	1		7	15
MN _S a ₂		11	1	4	5	17
NN _S S					-	2
NN _S a		2	1	1		6
NN _S a ₂		1		1	4	12
Total number of						
individuals with						
cDe(Rh ₀), V(hr ⁺)						
Fy(a-b-) or J ^s						
			7/64 (11%)			12/100 (12%)

able, the actual percentage of admixture in the hybrid populations has been calculated from frequencies of the African marker genes [28]. Quantitation of the African admixture in the contemporary Sicilian population is not feasible. However the frequencies of cDe(Rh₀) (3/), V(hr⁺) (7/), Fy(a-b-) (1/), and Js (3/), in the group of unrelated Sicilians suggest that the extent of admixture is comparable to that of other mixed Mediterranean and Middle Eastern populations with hemoglobin S [1, 8, 9, 21, 23].

Recent studies have indicated that the phenotype Fy(a-b-) is associated with resistance to malarial infection [16, 25]. The observation adds a new dimension to the generally accepted hypothesis that hemoglobin S has been selectively preserved in certain African and Mediterranean populations because of the protective effect of sickle cell trait against endemic malaria [2]. In the present survey we note that both Fy(a-b-) and hemoglobin S occur in native Sicilian populations who have resided in areas endemic for malaria until recent years. However the scope of the present study does not permit an assessment of the evolutionary interactions of Fy(a-b-), hemoglobin S and malaria.

In summary we observe that hemoglobin S is only one of multiple African genes present in contemporary Sicilian populations. Together with the historical evidence of population admixture, these findings support an African origin for the hemoglobin S gene in Sicilians. Accordingly we in-

Table II Blood groups of Sicilian families with sickle cell hemoglobin (Hb S) and of unrelated Sicilians with normal adult hemoglobin (Hb AA)

Phenotypes	Individuals from families with known Hb S					Unrelated native Sicilians Hb AA (n = 100)
	Hb AA (n = 4)	Hb AS (n = 21)	Hb SS (n = 5)	Hb A/ β thal (n = 11)	Hb S/ β -thal (n = 3)	
<i>ABO system</i>						
A ₁	2	4	1	4	4	29
A ₂	~	~	2	1	1	11
B	~	3	1	2	3	10
A ₁ B	~	~	~	~	~	6
A ₂ B	~	~	~	~	~	3
O	2	14	1	4	14	41
<i>Rhesus system</i>						
CCDee	2	6	~	2	5	21
CCDEe	~	~	~	~	~	3
CcDEe	2	4	~	2	5	16
CcDEeV	~	~	~	~	~	1
CcDee	~	7	4	3	8	3
CcDeeV	~	1	~	~	~	5
Ccddee	~	~	~	~	~	2
ccDEE	~	1	~	1	~	5
ccDee	~	1	~	1	~	2
ccDEe	~	~	1	~	4	4
ccDeeV	~	~	~	1	~	~
ccDEeV	~	~	~	~	~	1
ccdee	~	1	~	1	1	7
<i>Duffy system</i>						
Fy(a+b-)	1	2	3	1	4	1
Fy(a+b+)	2	11	2	6	13	43
Fy(a-b+)	~	8	~	4	4	36
Fy(a-b-)	1	~	~	~	~	1
<i>Kell system</i>						
Kk J _s (a-)	2	4	~	1	3	7
kk J _s (a-)	2	17	3	9	19	90
kk J _s (a+)	~	~	~	1	1	2
<i>MN system</i>						
MMSS	~	4	1	~	1	10
MMSS _h	2	2	1	3	5	3
MMss	1	~	~	2	1	12
MNSS	1	~	~	~	~	3

Table II (continued)

Phenotypes	Individuals from families with known Hb S					Unrelated native Sicilians Hb AA (n = 100)
	Hb AA (n = 4)	Hb AS (n = 1)	Hb SS (n = 5)	Hb A/ β -thal (n = 1)	Hb S/ β -thal (n = 2)	
<i>MN system</i>						
MN _{SA}		1	1		7	15
MN _{SS}		11	1	4	5	17
MN _{SS}						
NN _{SA}		2	1	1		6
NN _{SS}		1		1	4	12
Total number of individuals with cDe(Rh ₀), V(h ^r) Fy(a-b-) or J ^s						
			7/64 (11%)			14/100 (12%)

able, the actual percentage of admixture in the hybrid populations has been calculated from frequencies of the African marker genes [28]. Quantitation of the African admixture in the contemporary Sicilian population is not feasible. However the frequencies of cDe(Rh₀) (3 /), V(h^r) (7 /), Fy(a-b-) (1 /) and J^s (3 /) in the group of unrelated Sicilians suggest that the extent of admixture is comparable to that of other mixed Mediterranean and Middle Eastern populations with hemoglobin S [1, 8, 9, 21, 23].

Recent studies have indicated that the phenotype Fy(a-b-) is associated with resistance to malarial infection [16, 25]. The observation adds a new dimension to the generally accepted hypothesis that hemoglobin S has been selectively preserved in certain African and Mediterranean populations because of the protective effect of sickle cell trait against endemic malaria [2]. In the present survey we note that both Fy(a-b-) and hemoglobin S occur in native Sicilian populations who have resided in areas endemic for malaria until recent years. However the scope of the present study does not permit an assessment of the evolutionary interactions of Fy(a-b-), hemoglobin S and malaria.

In summary we observe that hemoglobin S is only one of multiple African genes present in contemporary Sicilian populations. Together with the historical evidence of population admixture, these findings support an African origin for the hemoglobin S gene in Sicilians. Accordingly we in-

terpret the occurrence of hemoglobin S in white persons of Sicilian ancestry to be the continuing dissemination of the original African mutation.

References

- 1 AKSOY M, IKEN, E., MOURANT A. E., and LEHMAN, H.. Blood groups, haemoglobins, and thalassemia in Turks in southern Turkey and Etr-Turks. *Br med. J* 11. 937-939 (1958)
- 2 ALLISON A. C. Parasitological reviews. Malaria carriers of the sickle-cell trait and in newborn children. *Expl Parasit.* 6 418-447 (1957)
- 3 BENZAD O, LEE, C. L. GAVIN J and MARSH, W. L.. A new anti-erythrocyte antibody in the Duffy system. *Anti-Fy^a Vox Sang* 24 337-342 (1973)
- 4 BERNSTEIN R. B. Mass screens for sickle cell disease. *J Am med. Ass.* 223 438 (1973)
- 5 BIANCO I, SCHILIRO, G., GRAZIANI, G. DE FEO, G. e SAPORITO, N.. Ricerca sulla frequenza di beta microcitemia e di emoglobine anormali nella popolazione della Sicilia sud-orientale. *Prog. Med.* 23 827 (1967).
- 6 BOYD, W. C.. Genetics and the human race. *Science* 140 1057-1064 (1963)
- 7 CANBY C. B. CARPENTER, G. and ELDREDGE, L. F. Drepanocytosis (sickleemia) and an apparently acute surgical condition of the abdomen. *Archs Surg.* 48. 123-125 (1944)
- 8 CHOREMBIS, C., IKEN, E. W., LEHMAN, H. MOURANT A. E., and ZANNOS, L.. Sickle-cell trait and blood groups in Greece. *Lancet* 11. 909-911 (1953)
- 9 CHOREMBIS, C., ZANNOS, L., IKEN E., MOURANT A. E., and LEHMAN, H. Blood groups of a Greek community with a high sickling frequency *Lancet* 11. 1333-1334 (1957).
- 10 CLARKE, F. Sickle-cell anemia in the white race with report of two cases. *Nebraska med. J* 18. 376 (1933).
- 11 DISTEFANO G. e MARCELLINI, M.. La determinazione dell'emoglobina A₂ mediante elettroforesi su acetato di cellulosa. *Riv. Pediat. sicroter* 24 36 (1944)
- 12 FOWLEY M. L. A history of Sicily Ancient Sicily to the Arab conquest (Chatto & Windus, London 1968)
- 13 GATTO, I. e RUSSO G. Risultati delle nostre osservazioni sulla drepanocitosi e talassodrepanocitosi. *Acta Genet. med. Gemell.* 8 179-194 (1959).
- 14 GELPI, A. P. Migrant populations and the diffusion of the sickle cell gene. *Ann. Intern. Med.* 79 258-264 (1973).
- 15 GELPI, A. P. Sickle cell trait and other African genetic markers. *J. Am. med. Ass.* 226 790 (1973).
- 16 GELPI, A. P. and KING, M. C.. Duffy blood group and malaria *Science* 191 1284 (1976)
- 17 GELPI, A. P. and PERRINE, R. P. Sickle cell disease and trait in white populations. *J. Am. med. Ass.* 224 605-608 (1973).
- 18 GREENWALD, L. and BURRETT J. B.. Sickle cell anemia in the white family *Am. J. Med. Sci.* 199 768-774 (1940)

- 19 GUYTON, R. A. and HERVEY, R. W. Sickle cell anemia in the white race: study of family with a review of genetic theories. *Am. J. Med. Sci.* 220: 272 (1950).
- 20 HADEN, R. L. and EVANS, F. D. Sickle cell anemia in the white race; improvement in two cases following splenectomy. *Archs Intern. Med.* 60: 133-142 (1937).
- 21 LEIDMAN, H. and CUTBUSH, M. Sub-division of some Southern Indian communities according to the incidence of sickle-cell trait and blood groups. *Trans. R. Soc. trop. Med. Hyg.* 46: 380-383 (1952).
- 22 LEIDMAN, H. and HENTENY, R. G. Man's haemoglobins, pp. 195-198 (Lippincott, Philadelphia 1966).
- 23 LEVINE, C., RACHOGLAWITZ, E. A., EZZIEL, M., FERNOLUCH, E., and SANDLER, S. G. Blood group phenotypes and hemoglobin S—an anthropologic study in two Israeli Arab communities. *Acta haemat.* 55: 300-305 (1976).
- 24 MCCRAW, C. J. Sickle cell trait in the white population. *J. Am. med. Ass.* 224: 1762-1763 (1973).
- 25 MILLER, L. H., MASON, S. J., CLYDE, D. F. and MCGOON, M. H. The resistance factor to *Plasmodium vivax* in blacks. The Duffy-blood-group genotype, *FyFy*. *New Engl. J. Med.* 293: 302-304 (1976).
- 26 MORRISON, M., SAWICKI, A. A. and LANDAUER, E. Sickle cell anemia in the white race: report of two cases with diagnosis by splenic puncture. *Am. J. Dis. Child.* 64: 881-887 (1942).
- 27 MOURANT, A. E., KOPEC, A. C., and DONAMICHELLA-SOMCZAK, K. Distribution of the human blood groups and other polymorphisms; 2nd ed., pp. 109-111 (Oxford University Press, London 1976).
- 28 POLLITZER, W. S. Problems in admixture estimates from different genetic loci. *Haematologica* 6: 193-198 (1972).
- 29 POWELL, W. N., RODARTE, J. G. and NIEL, J. V. The occurrence in family of Sicilian ancestry of the traits for both sickling and thalassemia. *Blood* 5: 887-897 (1950).
- 30 RACE, R. R. and SANDER, R. Blood groups in man, 6th ed., pp. 194-508 (Blackwell, Oxford 1975).
- 31 RAYEN, J. L. Haemoglobinopathies in Australia. *Med. J. Aust.* 2: 726-729 (1972).
- 32 SURIJANT, G. R. Geographical distribution of sickling in the clinical features of sickle cell disease, p. 29 (North-Holland, Amsterdam 1974).
- 33 SWENSON, E. C. T. Characteristics of African blood. *CRC Crit. Rev. clin. Lab. Sci.* 6: 19-45 (1975).
- 34 SMITH, D. M. A history of Sicily Medieval Sicily (800-1715)/modern Sicily (after 1715) (Chico & Windus, London 1968).
- 35 VALENTINO, L., LURA, V. Frequenza della stigmata 'falcemia' tra la popolazione infantile sana della Sicilia. *Pediatrics* 62: 547-551 (1954).
- 36 WEDDER, S. B. Sickle-cell anemia in an Italian child. *J. Mt. Sinai Hosp.* 4: 88 (1937).

Screening Coagulation Tests and Clotting Factors in Homozygous β -Thalassemia

L. CAOCCI, M. ALBERTI, P. BURRAI and R. CORDA

First University Department of Pediatrics,
Haemophilia and haemorrhagic disease Centre, Cagliari

Key Words. Contact coagulation factors Factor XI : Factor XII Cooley's anemia Homozygous β -thalassemia Kallikrein esterase Chronic hemolysis

Abstract In 30 children with homozygous β -thalassemia the hemostasis screening tests (bleeding time, PT PTT) platelet count and specific assays of clotting factors were carried out 25 days after their last transfusion. PT PTT and bleeding time showed minor variations, considerable thrombocytosis was found in splenectomized patients. Factors IX and XII were decreased in a high proportion of patients, the vitamin K-dependent factors (II, VII, IX, X) were slightly reduced and factors I V and VIII remained within the normal range in a majority of patients. Hepatic failure resulting in defective protein synthesis does not explain the more marked impairment of factors XI and XII, which might be secondary to activation of the intrinsic coagulation and/or kallikrein systems following intravascular haemolysis and multiple blood transfusions.

Introduction

Various coagulation abnormalities have been found in chronic hemolytic anemia such as sickle-cell anemia, paroxysmal nocturnal hemoglobinuria and hereditary spherocytosis [1-7]. An increase of factor VIII without significant variations of other coagulation factors and platelets was the most frequent finding. In sickle-cell anemia, a decrease of the contact phase coagulation factors (XI and XII) was shown by ABILDGAARD *et al* [1] during haemolytic crises this disease was also associated to a fall of

factor XIII accompanied by thrombocytopenia and subsequently followed by a rise in fibrinogen concentration and platelet count [10].

The β -thalassemia syndromes have rarely been studied in relation to the coagulation system. Although bruising and mucosal bleeding are quite frequent in the terminal phase of homozygous β -thalassemia, no marked deficiency of clotting factors was shown [9]. The function of the liver which plays a key role in the synthesis of coagulation factors in man, is known to be impaired in thalassemia [6, 8]. Hence, coagulation screening tests and specific assays of clotting factors were carried out in 30 children with homozygous β -thalassemia in order to evaluate the incidence of coagulation abnormalities and their possible role in the occurrence of bleeding symptoms.

Materials and Methods

30 children of both sexes with homozygous β -thalassemia (aged between 2 and 14 years) at different stages of the disease and with varying hemoglobin levels were included in this study (table I). Of the 30 patients, 5 had undergone splenectomy at an age varying from between 2 to 6 years. All the subjects received multiple blood transfusions: the transfusion regimen, which was usually begun in the first years of life and continued according to the severity of the disease, usually entailed transfusions of 2-3 U of packed red cells at intervals from 2 to 4 weeks.

The coagulation tests were performed at least 20-25 days after the last transfusion. Bleeding time was carried out by the method of Ivy *et al.* [11]. Platelet counts were done on EDTA venous blood by phase contrast microscopy according to BASCHIN and CLOUTTE [12]. For coagulation test, venous blood was drawn by venipuncture using plastic syringes and then transferred rapidly into plastic tubes containing 3.8% trisodium citrate $2H_2O$ (the amount of citrate was adjusted according to the hematocrit): it was then centrifuged at 3,000 g for 30 min at 4 °C, after which an aliquot of platelet-poor plasma was removed and stored in its until performance of the prothrombin time (PT), partial thromboplastin time (PTT), fibrinogen (F) and factor V and VIII assays were completed: aliquots were also frozen at -20 °C and stored for not more than 1 month before assaying the other factors.

PT, PTT, factor VIII and IX (one-stage assays) were performed using the methods described by DIXON [4]; fibrinogen by the method of VAMTLEN *et al.* [13], factor II with the one-stage method (Tulpan *enim*) of DIXON *et al.* [3]; factors V, VII and X with the one-stage methods described by DIXON [4]; factor XI and XII with the method of NISSELI [15].

The assay potencies (with the exception of fibrinogen) were related to reference pooled plasma, obtained from ten healthy volunteers (8 males and 2 females) and given an arbitrary potency of 100%. Such reference plasma was kept frozen at 20 °C and was not used for more than 15 days. For each clotting factor the normal range was established in 30 age-matched control children: the lower normal limit was calculated by subtracting 2 SD from the mean value.

Table 1 Coagulation screening tests, platelet count and Hb values in 30 children with homozygous β -thalassaemia

Patient No., Name	Age years	Hb g/dl	BT min	PT ratio	PTT ratio	Platelets mm ³
Normal range		14-16	3 min 10 sec- 5 min 40 sec	0.70-1.30	0.75-1.25	150,000- 400,000
1 M L.	2	11.6	6	1.10	0.98	353,000
2 M D.	2	6.5	4	1.11	0.97	220,000
3 C. M.	3	10.6	3.05	1.04	1.08	330,000
4 S. M. A.	3	11.2	4	1.01	1.03	575,000
5 A. P. P.	3	5.5	3.35	1.19	1.00	287,000
6 O. C.	4	10.4	3	1.08	1.06	385,000
7 O. G.	4	8.5	5.15	1.10	0.91	300,000
8 C. M. L.	5	10.3	4.20	1.22	1.01	270,000
9 F. D.	5	9.6	4.30	1.15	1.10	322,000
10 C. D.	5	6	8.30	1.28	1.30	140,000
11 D. N.	5	8.5	3.30	1.20	1.09	225,000
12 M. M.	5	4.5	6.20	1.12	1.05	151,000
13 M. M.	6	10.6	5	1.20	1.34	282,000
14 P. M.	6	10.8	6.45	1.13	1.19	123,000
15 D. A. P.	7	5.6	3.20	1.08	0.83	380,000
16 P. I.	7	10	5.30	1.23	1.14	204,000
17 M. B.	7	5.2	8.15	1.10	0.80	181,000
18 U. E.	8	7.9	4.10	1.21	1.30	158,000
19 P. A. P.	8	10.7	3.5	1.12	1.02	328,000
20 R. O.	9	10.2	4.25	1.12	1.03	298,000
21 P. R.	9	9.1	5.30	1.11	1.31	126,000
22 G. F.	10	8.8	5.50	1.09	1.04	218,000
23 R. G.	10	8.5	5.40	1.16	1.02	10,000
24 M. M. G.	11	12	3.30	1.09	1.16	246,000
25 T. F. M.	11	9.5	8.20	1.21	0.95	262,000
<i>After splenectomy</i>						
26 C. G.	10	7.8	2	1.26	1.27	430,000
27 S. S.	10	9.7	2.30	1.06	1.03	650,000
28 P. I.	11	8.7	4.30	1.30	1.30	960,000
29 P. C.	14	8	2.30	1.08	1.14	500,000
30 U. E.	14	7.1	5.10	1.06	0.96	560,000

Hb = Hemoglobin BT = bleeding time PT = prothrombin time PTT = partial thromboplastin time.

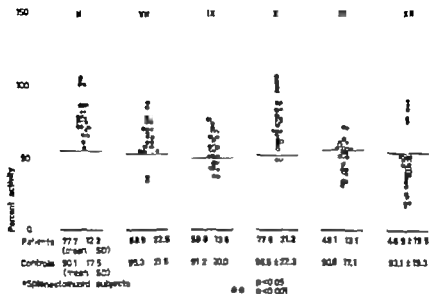


Fig 1 Coagulation factors II, VII, IX, XI and XII in 30 patients with homozygous β -thalassaemia. The horizontal line shows the lower limit of normal values.

Results

The results of the screening tests (bleeding time, platelet count, PT and PTT) were usually within the normal limits, established in our laboratory. Bleeding time was prolonged only in 3 of 30 patients the mean values were of 4 min 42 sec \pm 1 min 42 sec, against 4 min 30 sec \pm 1 min 30 sec in normal controls ($p > 0.6$). PT was slightly prolonged in 1 of 30 patients the mean ratio values were 1.14 ± 0.07 against 1 ± 0.12 in normal controls ($p < 0.001$). PTT was prolonged in 6 of 30 patients the mean ratio values were 1.08 ± 0.14 against 1.03 ± 0.03 in normal controls ($p > 0.05$). Platelet counts were slightly reduced in 3 patients, whereas 5 patients who had undergone splenectomy showed high platelet counts (430 000–960 000). The mean platelet count values were $322,460 \pm 180,730$ against $245 000 \pm 83,000 \times \text{mm}^3$ of normal controls ($p < 0.001$).

The mean values of fibrinogen (0.250 ± 0.050 g/dl) were similar to controls (0.268 ± 0.115 g/dl, $p > 0.3$). The mean values of factor V

Table 1 Coagulation screening tests, platelet count and Hb values in 30 children with homozygous β -thalassaemia

Patient No., Name	Age years	Hb g/dl	BT min	PT ratio	PTT ratio	Platelets mm ³
Normal range		14-16	3 min 10 sec- 5 min 40 sec	0.70-1.30	0.75-1.25	150,000- 400,000
1 M.L.	2	11.6	6	1.10	0.98	353,000
2 M.D.	2	6.5	4	1.11	0.97	220,000
3 C.M.	3	10.6	3.05	1.04	1.08	330,000
4 S.M.A.	3	11.2	4	1.01	1.03	575,000
5 A.P.P.	3	5.5	3.35	1.19	1.00	287,000
6 O.C.	4	10.4	3	1.08	1.06	385,000
7 O.G.	4	8.5	5.15	1.10	0.91	300,000
8 C.M.L.	5	10.3	4.20	1.22	1.01	270,000
9 F.D.	5	9.6	4.30	1.15	1.10	322,000
10 C.D.	5	6	8.30	1.28	1.30	140,000
11 D.N.	5	8.5	3.30	1.20	1.09	225,000
12 M.M.	5	4.5	6.20	1.12	1.05	151,000
13 M.M.	6	10.6	5	1.20	1.34	282,000
14 P.M.	6	10.8	6.45	1.13	1.19	123,000
15 D.A.P.	7	5.6	3.20	1.08	0.83	380,000
16 P.I.	7	10	5.30	1.23	1.14	204,000
17 M.B.	7	5.2	8.15	1.10	0.80	181,000
18 U.E.	8	7.9	4.10	1.21	1.30	158,000
19 P.A.P.	8	10.7	3.5	1.12	1.02	328,000
20 R.O.	9	10.2	4.25	1.12	1.03	298,000
21 P.R.	9	9.1	5.30	1.11	1.31	176,000
22 G.F.	10	8.8	5.50	1.09	1.04	218,000
23 R.G.	10	8.5	5.40	1.16	1.02	210,000
4 M.M.G.	11	12	3.30	1.09	1.16	246,000
25 T.F.M.	11	9.5	8.20	1.21	0.95	162,000
<i>After splenectomy</i>						
26 C.G.	10	7.8	2	1.06	1.27	430,000
27 S.S.	10	9.7	2.30	1.06	1.03	650,000
28 P.L.	11	8.7	4.30	1.30	1.30	960,000
29 P.C.	14	8	2.30	1.08	1.14	500,000
30 U.E.	14	7.1	5.10	1.06	0.96	560,000

Hb = Hemoglobin BT = bleeding time PT = prothrombin time PTT = partial thromboplastin time.

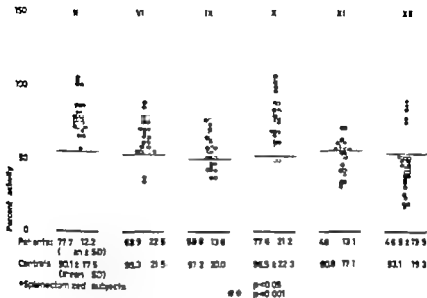


Fig. 1 Coagulation factors II, VII, IX, XI and XII in 30 patients with homozygous β -thalassaemia. The horizontal line shows the lower limit of normal values.

Results

The results of the screening tests (bleeding time, platelet count, PT and PTT) were usually within the normal limits, established in our laboratory. Bleeding time was prolonged only in 3 of 30 patients the mean values were of 4 min 42 sec \pm 1 min 42 sec, against 4 min 30 sec \pm 1 min 30 sec in normal controls ($p > 0.6$). PT was slightly prolonged in 1 of 30 patients the mean ratio values were 1.14 ± 0.07 against 1 ± 0.12 in normal controls ($p < 0.001$). PTT was prolonged in 6 of 30 patients the mean ratio values were 1.08 ± 0.14 against 1.03 ± 0.03 in normal controls ($p > 0.05$). Platelet counts were slightly reduced in 3 patients, whereas 5 patients who had undergone splenectomy showed high platelet counts (430 000–960 000). The mean platelet count values were $322,460 \pm 180,730$ against $245,000 \pm 83,000 \times \text{mm}^3$ of normal controls ($p < 0.001$).

The mean values of fibrinogen (0.250 ± 0.050 g/dl) were similar to controls (0.268 ± 0.115 g/dl $p > 0.3$). The mean values of factor V

Table 1 Coagulation screening tests, platelet count and Hb values in 30 children with homozygous β -thalassaemia

Patient No., Name	Age years	Hb g/dl	BT min	PT ratio	PTT ratio	Platelets mm ³
Normal range		14-16	3 min 10 sec- 5 min 40 sec	70-1.30	0.75-1.25	150,000- 400,000
1 M.L.	2	11.6	6	1.10	0.98	353,000
2 M.D.	2	6.5	4	1.11	0.97	220,000
3 C.M.	3	10.6	3.05	1.04	1.08	330,000
4 S.M.A.	3	11.2	4	1.01	1.03	575,000
5 A.P.P.	3	5.5	3.35	1.19	1.00	287,000
6 O.C.	4	10.4	3	1.08	1.06	385,000
7 O.G.	4	8.5	5.15	1.10	0.91	300,000
8 C.M.L.	5	10.3	4.20	1.22	1.01	270,000
9 F.D.	5	9.6	4.30	1.15	1.10	322,000
10 C.D.	5	6	8.30	1.28	1.30	140,000
11 D.N.	5	8.5	3.30	1.20	1.09	2.5 000
12 M.M.	5	4.5	6.20	1.12	1.05	151,000
13 M.M.	6	10.6	5	1.20	1.34	282,000
14 P.M.	6	10.8	6.45	1.13	1.19	123,000
15 D.A.P.	7	5.6	3.20	1.08	0.83	380,000
16 P.I.	7	10	5.30	1.23	1.14	204,000
17 M.S.	7	5.2	8.15	1.10	0.80	181,000
18 U.E.	8	7.9	4.10	1.21	1.30	158,000
19 P.A.P.	8	10.7	3.5	1.12	1.02	328,000
20 R.O.	9	10.2	4.25	1.12	1.03	298,000
21 P.R.	9	9.1	5.30	1.11	1.31	126,000
22 G.F.	10	8.8	5.50	1.09	1.04	218,000
23 R.G.	10	8.5	5.40	1.16	1.02	210,000
24 M.M.G.	11	1	3.30	1.09	1.16	46,000
25 T.F.M.	11	9.5	8.20	1.21	0.95	262,000
<i>After splenectomy</i>						
26 C.G.	10	7.8	2	1.26	1.27	430,000
27 S.S.	10	9.7	2.30	1.06	1.03	650,000
28 P.L.	11	8.7	4.30	1.30	1.30	960 000
29 P.C.	14	8	2.30	1.08	1.14	500,000
30 U.E.	14	7.1	5.10	1.06	0.96	560,000

Hb = Hemoglobin BT = bleeding time PT = prothrombin time PTT = partial thromboplastin time.

which are known to be earlier and more markedly affected in liver disease [5], were normal or less severely decreased.

WARDLE and PIERCE [19] have found elevated kallikrein-esterase plasma levels in association with reduced factor XII in a study of patients undergoing hemodialysis. Moreover ARILDGAARD *et al* [1] showed reduced factors XI and XII in sickle-cell anemia. We suggest that in homozygous β -thalassaemia chronic hemolysis and frequent blood transfusions determine an increased activity of kallikrein-esterase resulting in reduced levels of factors XII and XI. It appears unlikely that these abnormalities contribute to a great extent to the hemorrhagic tendency of patients with thalassemia, since decrease of factor XII is not associated with bleeding and the impairment of factor XI is too mild to account for it. Cutaneous bleeding is probably related to vascular defects caused by hypoxia, whereas gastrointestinal bleeding might be caused by portal hypertension and splenomegaly.

Acknowledgement

We would like to thank Prof. M. M. MANOUCCI for helpful criticism and advice.

References

- 1 ARILDGAARD, C. F. SØRENSEN, J. V. and SCHULMAN, I. Factor VIII (antihemophilic factor) activity in sickle-cell anemia. *Br J Haemat* 19 19-27 (1967).
- 2 BRICKER, J. G. and CROCHITT, E. P. Morphology and enumeration of human blood platelets. *J appl. Physiol* 3 363-377 (1950).
- 3 DENSON, K. W. E., ROBERTS, R., and BROS, R. The specific assay of prothrombin using the Taipan Snake Venom. *Br J Haemat* 21 219-226 (1971).
- 4 DENSON, K. W. E., in BROS Human blood coagulation, haemostasis and thrombosis (Blackwell, Oxford 1976).
- 5 DEUTCH, E., Blood coagulation changes in liver disease. *Prog. Liver Dis.* 2 69-83 (1965).
- 6 ECONOMIDOU, J. CONSTANTOULAKIS, M. AUGUSTAKIS, O. T. YLOS, P. E., ZUCKERMAN, A. J., BADGES, P. M., and BEYCEPOV, M. A., The incidence of hepatitis-associated antigen and antibody in patients with thalassemia in Greece. *Vox Sang* 19 401-403 (1970).
- 7 ESKERUD, O., Blood coagulation and intravascular hemolysis. *Scand. J. clin. Lab. Invest.* 14 217-222 (1962).
- 8 GRAUD, P. ORSINI, A. et MURATORE, R. Le foie dans la maladie de Cooley. *Pédiatrie* 14 125-142 (1959).

were $75.4 \pm 14.6\%$ (controls $85.6 \pm 24.5\%$ $p > 0.05$) and those of factor VIII were $82.7 \pm 31.6\%$ (controls $97.1 \pm 28.2\%$ $p > 0.05$)

Figure 1 shows the values for the vitamin K-dependent clotting factors II VII IX, X there was no significant difference from the control group, except for factor IX which was lower than the normal limit in 9 of 30 cases (30%) with a significantly different mean value ($58.8 \pm 13.6\%$ $p < 0.05$) Factor XI was decreased in 17 of 30 cases (57%) and factor XII in 24 cases (80%) (fig 1) Mean values of both factor XI (49.1 ± 13.1 $p < 0.001$) and factor XII (46.9 ± 19.5 $p < 0.01$) were significantly lower than those of the control group There was no significant correlation between the values factors and hemoglobin values. Abnormalities were also found not to be related to age, number of transfusions or splenectomy

Discussion

From these results it appears that the hemostatic system is not severely impaired in β -thalassemia. Platelet count always gave almost normal results in the group of patients who had not undergone splenectomy while postsplenectomy thrombocytosis was probably related to the absence of the spleen function Since bleeding time was normal in the majority of patients, a platelet qualitative defect could also be ruled out. As for the coagulation system contact phase factors XI and XII were more markedly reduced than vitamin K-dependent factors (II VII IX X) whereas fibrinogen and factor V were found to be normal Unlike other hemolytic anemias [1 7 12] factor VIII was not increased this is probably related to the fact that in thalassemia major the underlying factors determining factor VIII increase (endothelial damage and massive intravascular hemolysis) are not so prominent as in sickle-cell anemia, spherocytosis, paroxysmal nocturnal hemoglobinuria and favism [1 7 10 17]

The contact phase factors XI and XII constitute the first center of activation in the intrinsic pathway of blood coagulation and in turn activates factors IX and VIII Factor XII (Hageman factor) is also known to play a key role in the activation of the kinin system fibrinolysis and platelets [5 16 20] Although contact factors are thought to be synthesized by the liver [16-18] the impairment of this organ in thalassemia is unlikely to be the most important mechanism of the decreased levels found in these patients. In fact the vitamin K-dependent factors and factor V

A. ENCKELHARDT und H. LÖNNEL. Diagnostik hämorrhagischer Diathesen. Methodische Fortschritte im medizinischen Laboratorium, vol. 4. Verlag Chemie, Weinheim 1977. VIII + 204 pp. 68 fig., 34 tab., DM 58,-.

Von einem Buch über Fortschritte, erschienen 1977, darf der Leser Newigkeiten bis Mitte 1976 erwarten. Es ist deshalb enttäuschend, dass nur das letzte (7.) Kapitel eine übrige gelungene Besprechung der Gerinnungsdiagnostik mit chromogenen Peptid-Substraten (WITT, Freiburg i. Br.) - der Erwartung entspricht. Im ersten Kapitel - einer hervorragenden Darstellung der Grundlagen der Hämostaseologie (MÜLLER-BERCHAU, Gießen) - trägt die jüngste Referenz die Jahreszahl 1972. I bring auf Indikationsstellung zur hämostaseologischen Analyse (zweites Kapitel) stört der Mangel an Aktualität natürlich weniger. Aber im dritten Kapitel (Angeborene und erworbene Kongenopathien) vermisst man im sehr eruditen Beitrag über angeborene Störungen (LECHEL, Köln) die Biochemie der Fletcher und der Williams- bzw. Fitzgerald- und Fanconi Kongenopathie sowie die heutigen Ansichten über die Beziehungen zwischen dem Gerinnungs- dem Komplement- dem Kalikrein- und dem Kininsystem. Auch fehlen Hinweise auf das Vorkommen eines schweren Faktor VII-Mangels ohne hämorrhagische Diathese und auf die schon 1970 beschriebene, überraschende Verlasto ersthafter Hämophilie B, die mit zunehmendem Alter der Patienten praktisch verschwindet. Vergeblich sucht man nach Angaben über die vor allem in den letzten Jahren ganz wesentlich verbesserte Diagnostik von Überträgerinnen der Hämophilie A. Im recht schwerfälligen geschriebenen Teil über erworbene Kongenopathien (OULEN, Bialist) sollte anstelle von mühsigen Wiederholungen und wenig instruktiven Tabellen und Abbildungen der bei Amyloidose in schweren Fällen vorkommende, schwere selektive Faktor X-Mangel eingehender beschrieben werden, und die schon vor 5 Jahren erkannte Abwesenheit einer hämorrhagischen Diathese bei Patienten mit deutlich verzögerter Gerinnung durch ein zirkulierendes Antithromboplastin bei Lupus erythematosus und ähnlichen Krankheitszuständen sollte gebührend erwähnt werden. Im vierten Kapitel (Thrombozytäre Gerinnungsstörungen) gehen in der nachsthaft geschriebenen Übersicht über thrombozytäre Störungen der primären Hämostase (HÄRZOG, Gießen) die Literaturhinweise nicht weiter als bis 1974. Und im fünften Kapitel (Antikoagulantien) sucht der interessierte Leser vergeblich nach dem jüngsten Stand der Prothrombinstandardisierung. Das sechste Kapitel schließlich (Qualitätskontrolle gerinnungsphysiologischer Untersuchungsverfahren) ist mit seinem einzigen Beitrag (BAUM, Bonn) nicht mehr als ein gut gelungener Ansatz zur Diskussion der nach 1975 (jüngste Referenz) sich schnell entwickelnden Aktivitäten.

Zusammenfassend lässt der vorliegende Band in Bezug auf Aktualität sehr zu wünschen übrig. Es ist den Herausgebern offensichtlich nicht gelungen, die Beiträge der 37 Mitarbeiter zu synchronisieren. Begrüßlich, aber nicht weniger störend, ist die unterschiedliche Qualität der Beiträge. Neben den erwähnten Übersichten sind die folgenden methodologischen Teile besonders gut gelungen: Global- und Grup-

- 9 HILGARTNER, M. and SMITH, C. H. Coagulation studies as a measure of liver function in Cooley's anemia. *Ann. N Y Acad. Sci.* 119 631-640 (1964).
- 10 ITTYRAH, R., ALKJAERSEN, N. FLETCHER, A., and CHAPLIN, H. Coagulation factor XIII concentration in sickle-cell disease. *J Lab. clin. Med.* 88. 546-554 (1976).
- 11 IVY A. C., NELSON D. and BUCHER, G.. The standardization of certain factors in the cutaneous 'venostasis bleeding time technique. *J Lab. clin. Med.* 83. 1812-1818 (1940).
- 12 MANGUCCI, P. M. LONNA, G. F. CAOCCI, L., and DROGUARDI, N.. Effect on blood coagulation of massive intravascular haemolysis. *Blood* 33 207-213 (1969).
- 13 MEIER, H. L. PIERCE, J. V., COLMAN, R. W. and KAPLAN, A. P.. Activation and function of human Hageman factor *J clin. Invest.* 60 18-31 (1977).
- 14 MURPHY R. C. and SHAPIRO, S.. The pathology of sickle-cell disease. *Ann. intern. Med.* 23 376-387 (1945).
- 15 NOSSEL, H. L. The contact phase of blood coagulation (Blackwell, Oxford 1976).
- 16 RAPAPORT S. L. Plasma thromboplastin antecedent levels in patients receiving coumarin anticoagulant and in patients with Laennec cirrhosis. *Proc. Soc. exp. Biol Med.* 103 115-116 (1961).
- 17 ROBERTS, R. H. and CEDERBAUM, A. I. The liver and blood coagulation. Physiology and pathology *Gastroenterology* 63 297-320 (1972).
- 18 VERMYLEN, C. VREIER, R. A. DE, and VERSTRAETE, M.. A rapid enzymatic method for assay of fibrinogen. fibrin polymerization time (FPT test). *Chim. clinica Acta* 8 418-424 (1963).
- 19 WARDLE, E. M. and PIERCE D. A.. Studies of contact activation of blood in haemodialysis. *J clin. Path.* 25 1045-1049 (1972).
- 20 WEISS, A. S. GALLIN, J. I., and KAPLAN, A. P. Fletcher factor deficiency A diminished rate of Hageman factor activation caused by absence of prekallikrein with abnormalities of coagulation fibrinolysis, chemotactic activity and kinin generation. *J clin. Invest.* 53 622-633 (1974).

A. ENGELMANN und H. LONELLI: Diagnostik hämorrhagischer Diathesen. Methodische Fortschritte im medizinischen Laboratorium, Bd. 4. Verlag Chemie, Weinheim 1977. VIII + 204 pp., 68 fig., 34 tab., DM 58,-.

Von einem Buch über Fortschritte, erschienen 1977 darf der Leser Neugierden bis Mitte 1976 erwarten. Es ist deshalb enttäuschend, dass nur das letzte (7.) Kapitel – eine übrigens gelungene Besprechung der Gerinnungsdiagnostik mit chromogenen Peptid-Substraten (WITT, Freiburg i. Br.) – der Erwartung entspricht. Im ersten Kapitel einer hervorragenden Darstellung der Grundlagen der Hämostaseologie (MÜLLER-BRECHAU, Gießen) – trägt die jüngste Referenz die Jahreszahl 1975. In bezug auf Indikationsstellung zur hämostaseologischen Analyse (zweites Kapitel) stört der Mangel an Aktualität natürlich weniger. Aber im dritten Kapitel (Angeborene und erworbene Koagulopathien) vermisst man im sehr eruditen Beitrag über angeborene Störungen (LACHNER, Köln) die Biochemie der Fletcher- und der Williams- bzw. Fitzgerald- und Fungiec-Koagulopathie sowie die beständigen Ansichten über die Beziehungen zwischen dem Gerinnungs- dem Komplement dem Kalikrein- und dem Kininsystem. Auch fehlen Hinweise auf das Vorkommen eines schweren Faktor VII-Mangels ohne hämorrhagische Diathese und auf die schon 1970 beschriebene, überraschende Variante ernsthafter Hämophilie B, die mit zunehmendem Alter der Patienten praktisch verschwindet. Vergleichlich sucht man nach Angaben über die vor allem in den letzten Jahren ganz wesentlich verbesserte Diagnostik von Überträgerinnen der Hämophilie A. Im recht schwerfälligen geschriebenen Teil über erworbene Koagulopathien (OHLER, Mielitz) sollte anstelle von unnötigen Wiederholungen und wenig instruktiven Tabellen und Abbildungen der bei Amyloidose in seltenen Fällen vorkommende, schwere erbliche Faktor X Mangel eingehender beschrieben werden, und die schon vor 5 Jahren erkannte Abwesenheit einer hämorrhagischen Diathese bei Patienten mit deutlich verzögerter Gerinnung durch ein zirkulierendes Antithromboplastin bei Lupus erythematosus und ähnlichen Krankheitszuständen sollte gebührend erwähnt werden. Im vierten Kapitel (Thrombozytäre Gerinnungsstörungen) gehen in der mehrheitlich geschriebenen Übersicht über thrombozytäre Störungen der primären Hämostase (HARZMAN, Gießen) die Literaturberichte nicht weiter als bis 1974. Und im fünften Kapitel (Antikoagulation) reicht der interessierte Leser vergleichlich nach dem jüngsten Stand der Prothrombinzeitstandardisierung. Das sechste Kapitel schließlich (Qualitätskontrolle gerinnungsphysiologischer Untersuchungsmethoden) ist mit seinem einzigen Beitrag (BRUNN, Bonn) nicht mehr als ein gut gelungener Ansatz zur Diskussion der nach 1975 (jüngste Referenz) sich schnell entwickelnden Aktivitäten.

Zusammenfassend lässt der vorliegende Band in bezug auf Aktualität sehr zu wünschen übrig. Es ist den Herausgebern offensichtlich nicht gelungen, die Beiträge der 11 Mitarbeiter zu synchronisieren. Begreiflich, aber nicht weniger störend, ist die unterschiedliche Qualität der Beiträge. Neben den erwähnten Übersichten sind die folgenden methodologischen Teile besonders gut gelungen: Global- und Grup-

pentests (BECKER, Bonn) Fibrinogen (PAAR, Essen) Thrombozytenzählung in der täglichen Routine (WEINAND, Krefeld) und die Laboratorienkontrolle der Heparinothérapie (HILKE und OSTENDORF Tübingen).

Die immunologischen Methoden in der Gerinnung (HEIDENROTH und KARGES, Marburg/Lahn letzte Referenz 1974!) betreffen eine ausgezeichnete Einführung zum Studium dieser neuen Untersuchungstechnik; zu wenig wird jedoch hingewiesen auf das für immunologische Methoden vorläufig beschränkte Anwendungsgebiet im klinisch-diagnostischen Labor. Übrigens trägt eine ganze Reihe methodologischer Beiträge allzu stark den Charakter von teils wenig kritischen Originalarbeiten. Der vorliegende Band über methodische Fortschritte in der Diagnostik hämorrhagischer Diathesen beweist einmal mehr wie ausserordentlich schwierig es ist, Bleibendes und für die Praxis Wichtiges von nur teilspezialistisch experimentell Interessantem zu trennen. Wie schnell auch die Methodologie im Labor sich zu verändern scheint, das nützliche Rüstzeug für eine diagnostisch erfolgreiche Laboratoriumspraxis verändert sich nur langsam.

E. A. LOELIGER, Leiden

J. C. ELLORY and V. L. LEW (eds) *Membrane Transport in Red Cells*. Academic Press, London 1977. X + 470 pp. £ 21.00. ISBN 0-12 237150-2.

This is not one of those volumes collecting odd ends of 'latest progress' brought together haphazardly in a specialists' meeting, but a well-balanced text made up of 18 review type essays written by the very best workers in the respective fields. The editors' competence is reflected not only by their wise selection of the contributors but also by the four excellent monographs written by themselves for the book. All aspects of the transfer of water and solutes across the red cell membrane are dealt with adequately (inorganic ions, pH equilibration, organic anions, choline, amino acids, sugars, glutathione). In addition to normal human erythrocytes, genetically abnormal human red cells and the normal physiology of those from ruminants, birds and cat and dog are considered. The book is addressed to the physiologist for whom the red cell, dead as it may be, is an exciting toy in which a host of fundamental questions pertaining to membrane function can be put to test. The book, therefore, takes the reader to the forefront of research. However the newcomer is not taken aback by a technical fireworks cracked off in darkness but patiently led into the wonderland of membranology. There cannot be any doubt that a thorough understanding of the basic physiology not only of oxygen transport by red cells but also of their permeability properties is necessary to elucidate a good number of pathologic deviations. It is for this reason that the present book is of very much concern for clinical haematologists, too.

H. J. SCHIATZMAN, Bern

J. V. DACE and S. M. LEWIS *Practical Haematology*, 5th ed. Churchill/Livingstone, London 1975. VIII + 629 pp., £ 6.00. ISBN 0-443-01262-8.

Prof. DACE's 'Practical Haematology' was first edited in 1950. Throughout later editions, the author has been joined by several co-authors, a fact reflecting the increasing complexities of haematology. However even the latest edition of this out-

standing reference book has remained remarkably concise, despite the fact that important developments of cell biology protein chemistry and immunology have been faithfully recorded.

It is always a matter of personal opinion which laboratory method should be recommended. All practical methods which have been included into this book are described in such detail that even a non-specialized technician, by following it to the letter should be capable of reproducing it. An appendix on common buffer solutions, reagents, standardization procedures, units, etc., is most welcome. The bibliography is adequate within the scope of the book. A fairly complete index has been found to be helpful.

Finally I apologize for reviewing this remarkable book with some delay. Meanwhile, its value as a standard reference book for the haematology laboratory has not been surpassed.

E. A. BUCK, *Bonn*

H. F. BURN, B. FORCET and H. RAJCHET: *Human Hemoglobins*. Saunders, Philadelphia 1977. 432 pp., £ 17.00 ISBN 0-7216-2178-1.

In this book most competent authors give a complete review of hemoglobin structure, functional properties of hemoglobin, oxygen and carbon dioxide transport in red cells, hemoglobin biosynthesis, the thalassemias, human hemoglobin variants, sickle cell anemia and related disorders, unstable hemoglobin variants, mutants with abnormal oxygen binding, M hemoglobins, methemoglobinemia and carboxyhemoglobinemia. Valuable new information is given on the pathophysiological mechanisms and the resulting clinical disorders. Over 280 hemoglobin variants are listed in synoptical tables. The reader is referred to more than 1,800 references. This book can be recommended to laboratory investigators and clinicians. It should be disposable in a hospital library.

H. R. MARTI, *Aarau*

A. KACK, H. RADECKY, H. THOMAS and K. WINTER (Hrsg.): *Leitfaden des Transfusionswesens*. Schriftenreihe der Akademie für Ärztliche Fortbildung der DDR, Band 43. Verlag Volk und Gesundheit, Berlin 1977. 304 pp., 29 fig., 53 tab. M 37.-

In a series of booklets published by the Akademie für Ärztliche Fortbildung der DDR this book deals with blood transfusion and transplantation immunology. 15 authors working in these fields and well recognized in their country have written valuable contributions. The booklet has been divided into 15 chapters which cover all aspects of blood transfusion: organization, indication for blood transfusion, artificial volume substitutes, techniques of blood transfusion, serological problems involved in blood transfusion, side effects, haemolytic diseases of the newborn, and immunology of organ transplantation. Though short and limited to essential matters, the various chapters contain a considerable amount of information. Moreover the booklet provides an excellent survey on the blood bank situation in the German Democratic Republic.

S. SAUER, *Frankfurt/Main*

R. K. ARCHER and L. B. JEFFCOTT (eds.) *Comparative Clinical Haematology* Blackwell, Oxford 1977 737 pp. £ 25.00. ISBN 0-632-00289-1.

This comparatively large (737 pages) book is composed of 13 chapters dealing with the haematology of exotic mammals, horses, oxen, pigs, sheep, goats, deer, dogs, cats, birds, rats, mice, marmosets and – quite aptly – man. As Prof. LEHMANN states in his foreword the various surveys are masterful, and each one is complete in itself. Obviously the book is a must for the researcher performing haematologic investigations in animals and for the scientifically minded veterinarian. Also, the basic general haematologist – if such a generalist exists – will be interested in the common patterns of blood diseases though the different species, and in the fascinating aspects of phylogenesis of blood. However if a clinical haematologist, such as this reviewer is asked to comment on this book, he will be bound to follow the opposite route, and to privilege the information flowing back from animal to human haematology. In this spirit, a few words need to be said on S. M. LEWIS' outstanding synthesis of the haematology of man. It seems quite impossible how much authoritative information is made to be packaged in only 77 pages, it is only a pity that some photomicrographs do not compare favourably with the text's excellency.

Going on to the following sections, anyone who has witnessed the wicked wonder of sickling under the microscope will be fascinated by the non-wicked sickling of the deer here again due to 'different (although non-pathological) haemoglobins, and quite paradoxically produced by oxygenation and reversed by bubbling carbon dioxide. The equally fascinating canine SLE is briefly discussed, even if devoid of some of its most recent and provocative findings. The final technical chapter is invaluable for veterinarian haematology but may prove a good reminder for the clinician.

All in all, *Comparative Clinical Haematology* is to be regarded as an outstanding contribution to the better knowledge of blood and blood diseases in the animal kingdom, without excepting man.

A. M. MARCANTO Genova

R. L. LUNDELAD, J. W. FENTON, II and K. G. MANN: *Chemistry and Biology of Thrombin*. Science Publishers, Ann Arbor Mich. 1977 XII + 564 pp., US\$ 24.75. ISBN 0-250-40160-6.

This book contains proceedings of the Conference on the Chemistry and Biology of Thrombin which was held at the Mayo Clinic in March/April 1977. The introducing presentations cover the methods for isolation and characterization of thrombin. Biological and biochemical properties of various thrombin preparations are described and compared with those of thrombin-like enzymes from snake venoms and other serine proteases. Thrombin structure and the conformation of the active site are investigated by advanced physicochemical methods such as X-ray diffraction, electron spin resonance, fluorescence and affinity labeling. Several contributors discuss the kinetics of prothrombin activation *in vitro* and of thrombin reactions with substrates and inhibitors.

Biological functions of thrombins are involved in blood coagulation therefore, special attention is devoted to the fibrinogen-fibrin conversion and to the thrombin-induced activation of the clotting factors V, VIII and XIII. In addition, interac-

tions of thrombin with complement factors C3 and C5, phospholipid vesicles, platelets, and fibroblasts are examined. Influence of thrombin on cell proliferation is another subject of communications presented in these proceedings. Finally several authors present their results concerning the regulation of thrombin activity in the circulation by physiological inhibitors, such as heparin, antithrombin III and α_1 -proteinase inhibitor. All papers are accompanied by reference list and subject index is included. This book will serve as an information source to biochemists interested in fundamental research, and also to hematologists and clinicians working in the field of hemostasis.

M. FURLAN, Bern

G. GORDON STEEL. *Growth Kinetics of Tumours*. Clarendon Press, Oxford 1977 307 p.

This excellent book includes the following chapters: Growth rate of tumours, Basic theory of growing cell population, Experimental techniques for cell kinetic studies, Technique of labelled tumours, Cell population kinetics of tumours in experimental animals, Cell population kinetics of human tumours, Growth and survival of tumour stem cells, Cell population kinetics and therapeutic response. Much basic information can be found in this 307-pages volume. For the physician, the last chapter is particularly interesting. A very large bibliography (33 pages) is very valuable complement to this book. A glossary of the terms used in cell kinetics studies can be useful to some readers. This book should be bought by every library and scientist interested in fundamental studies in cancerology.

Y. NAIZAK, Paris

U. MÖLLER-ESTERHARD, P. A. MIESCHER and E. R. JAFFÉ (eds): *Iron Excess. Alterations of Iron and Porphyrin Metabolism*. Grune & Stratton, New York 1977 VIII + 370 pp., US\$ 22.50. ISBN 0-8089-1040-X

This volume assembles two issues of *Seminars in Hematology* which also appeared in 1977. Competent groups of authors review current concepts of the chemistry of iron, human iron metabolism in general and pertinent aspects of iron overload. Understanding of the mechanisms involved in iron storage and transport and in regulation of heme synthesis forms the basis for insight into pathological conditions. Consequently structure and function of ferritin and transferrin are thoroughly discussed with particular reference to functional implications of structural heterogeneity of the ferritin molecule and the two transferrin iron-binding sites. Besides overviews of iron overload in adult and pediatric medicine, and critical considerations on chelation therapy special emphasis is put on disorders of porphyrin metabolism. The position of strict phlebotomy therapy in classical hemochromatosis and in porphyria cutanea tarda is again strengthened. A carefully edited monograph presenting both biochemical and clinical information of the highest possible standard.

A. GÄRNER, Ulm

Index rerum ad Vol. 60

Bearbeitet von G. Boerni, Basel

(B) = Book reviews Buchbesprechungen Livres nouveaux

- Aberrations of iron and porphyrin metabolism. Iron excess 369 (B)
- Absorption of iron, v Food iron absorption
- Acetylcholinesterase erythrocyte acetylcholinesterase activity changes in the early stages of anemic and hypobaric hypoxia or after CoCl_2 treatment in rabbits 21
- Achylia gastrica inorganic iron absorption in subjects with iron deficiency anemia, achylia gastrica and alcoholic cirrhosis using a whole-body counter 182
- Acid base balance, v Hypoxia
- Acid dialysis, v Folate binder
- Acute and chronic myelofibrosis (A retrospective study) 108
- Adenosine diphosphate, v Platelet aggregation
- Adrenergic beta-receptor blockade peripheral platelet count in response to salbutamol before and after beta-receptor blockade 310
- African admixture, v Blood group phenotypes (*Sicklows*)
- Aggregation of platelets in diabetic retinopathy 53
- Agranular metamorphosis of chronic myelogenous leukaemia, lymphoid antigens on blast cells 280
- Alcoholic cirrhosis inorganic iron absorption in subjects with iron deficiency anemia, achylia gastrica and alcoholic cirrhosis using a whole-body counter 182
- Alkylating agents, v Paraproteinaemia
- Allogeneic bone marrow transplantation methotrexate and citrovorum factor after histoincompatible allogeneic bone marrow transplants in dog 233
- Alpha-chain interaction between iron deficiency and α -thalassaemia the *in vitro* effect of haemin on α -chain synthesis 1
- Alpha-thalassaemia interaction between iron deficiency and α -thalassaemia the *in vitro* effect of haemin on α -chain synthesis 1
- Amino groups of the human platelet membrane 304
- Anaemia, Cooley's anaemia, v Thalassemia
- Anaemia, haemolytic, v Leukaemia, lymphocytic, chronic (in *Israel*)
- Anaemia, hypoplastic, congenital, refractory to corticosteroids but responding to cyclophosphamide and antilymphocytic globulin (Report of a case having responded with a transitory wave of dyserythropoiesis) 90
- Anaemia, iron deficiency inorganic iron absorption in subjects with iron deficiency anemia, achylia gastrica and alcoholic cirrhosis using a whole-body counter 182

- ; platelet counts in children with iron deficiency anaemia 85
- v Iron and zinc absorption
- Anaemia, mediterranean, Thalassaemia
- Anaemia, refractory: clinical contribution to the knowledge of haemopoietic dysplasias: long-term follow-up of 13 patients with refractory anaemia 10
- Anaemic and hypobaric hypoxia: erythrocyte acetylcholinesterase activity changes in the early stages of anaemic and hypobaric hypoxia or after CoCl_2 treatment in rabbits 31
- Analysis, discriminant, on serum and bone marrow parameters, Monoclonal gammopathies
- Anemia, hemolytic, Leukemia, lymphocytic, chronic (in *Arab*)
- Iron and zinc absorption
- Anemia, iron deficiency: inorganic iron absorption in subjects with iron deficiency anaemia, achylia gastrica and alcoholic cirrhosis using a whole-body counter 182
- platelet counts in children with iron deficiency anaemia 85
- Anemia, refractory: clinical contribution to the knowledge of hemopoietic dysplasias: long-term follow-up of 13 patients with refractory anemia 10
- Anemic and hypobaric hypoxia: erythrocyte acetylcholinesterase activity changes in the early stages of anemia and hypobaric hypoxia or after CoCl_2 treatment in rabbits 21
- Antigen, lymphoid, on blast cells in the agranular metamorphosis of chronic myelogenous leukaemia 280
- Antilymphocytic globulin: congenital hypoplastic anaemia, refractory to corticosteroids but responding to cyclophosphamide and antilymphocytic globulin (Report of case having responded with a transient wave of dyserythropoiesis) 90
- Antilymphocytic globulins, v Antigens, lymphoid
- Antineoplastics, v Busulfan, Cyclophosphamide, Methotrexate
- Aplasia of red cells (adult, pure): paraproteinemic variety of pure red cell aplasia (Immunological studies on 1 patient) 227
- Arab*, *Small Arab*
- Arachidonic acid, v Platelet aggregation
- Autoimmune disorder v Red cell aplasia (adult, pure)
- Autoradiography MAY HEMOLYSE anomaly
- Bence Jones proteinuria, "idiopathic" 269
- Bence Jones proteinuria (subtype K): chronic granulocytic leukemia, neutrophilic type, with paraproteinemia (IgA type K) 316
- Benign monoclonal gammopathies, Gammopathies
- Beta-receptor blockade: peripheral platelet count in response to salbutamol before and after adrenergic beta-receptor blockade 310
- Beta-thalassaemia: serum lipid pattern 100
- screening coagulation tests and clotting factors in homozygous β -thalassaemia 358
- in Sickle (Hematological and biophysical studies) 193
- Binding protein: folic acid binding protein in chronic granulocytic leukaemia: the effect of methotrexate 341
- Bending of protoporphyrin and haem in to human spectrin 321
- Biology and chemistry of thrombin 368 (B)
- Biosynthesis, v Alpha-chain
- Biosynthesis of hemoglobin, v Beta-thalassaemia
- BLACKFAN, DIAMOND
- Blast cells: lymphoid antigens on blast cells in the agranular metamorphosis of chronic myelogenous leukaemia 280
- Blast transformation of lymphocytes, Lymphocyte transformation tests
- Blood coagulation, v Coagulation tests, Haemorrhagic Diatheses (B), Fibrinogen activator, Platelet aggregation, Thrombin (B), Thrombosis (B)

- Blood group phenotypes and the origin of sickle cell hemoglobin in *Stellans* 350
- Blood transfusion v Transfusion (B)
- Blutgruppen, v Blood group phenotypes
- Bluttransfusion, v Transfusion (B)
- Bone marrow cell suspensions, iron uptake, v Iron uptake by cell suspensions
- Bone marrow cells cytoplasmic immunoglobulins in bone marrow cells of polyclonal and of monoclonal origin 137
- Bone marrow granulocyte reserve, defect in liver cirrhosis, evaluated with etiocholanolone 291
- Bone marrow parameters differentiation between benign and malignant monoclonal gammopathies by discriminant analysis on serum and bone marrow parameters 129
- Bone marrow transplants methotrexate and citrovorum factor after histoincompatible allogeneic bone marrow transplants in dog 233
- Book reviews 62-64 (B) 126-128 (B) 365-369 (B)
- B (T) lymphocytes, v Red cell aplasia (adult, pure)
- B (T) rosetting lymphocytes, v Mouse red cell
- Buchbesprechungen 62-64 (B), 126-128 (B), 365-369 (B)
- Busulphan, v Megakaryocytic leukaemia
- Canis*, v Dog
- Canine leukocytes, v Colony formation (in vitro)
- ^4C -5CH₃H pteroylglutamic acid, v Folate binder
- Cell cultures, v Colony formation, Iron uptake by cell suspensions (fibroblasts, lymphoblasts)
- Cell electrophoresis, v Platelet membrane
- Cell suspensions from bone marrow and liver v Iron uptake by cell suspensions
- Chain, α -chain interaction between iron deficiency an α -thalassaemia the *in vitro* effect of haemin on α -chain synthesis 1
- Chemistry and biology of thrombin 368 (B)
- Chemotherapy v Non-Hodgkin's lymphoma
- Children platelet counts in children with iron deficiency anemia 88
- Children, *Turkish* children decreased iron and zinc absorption in *Turkish* children with iron deficiency and geophagia 76
- Chlorpromazine, v Red cell flexibility
- Chromatography v Folate binder Folic acid binding
- Chromosome, Philadelphia chromosome, v Lympho-myeloid complex
- Chromosomes, v Refractory anemia
- Chronic hemolysis, v Coagulation tests
- Chronic leukemias (Clinica in haematology Vol. 6, Nr 1) 64 (B)
- Cirrhosis of the liver defect of bone marrow granulocyte reserve in liver cirrhosis evaluated with etiocholanolone 291
- v Alcoholic cirrhosis
- Citrovorum factor and methotrexate after histoincompatible allogeneic bone marrow transplants in dogs 233
- 'Classic and 'acute myelofibrosis (A retrospective study) 108
- Clinical contribution to the knowledge of hemopoietic dysplasias long-term follow-up of 13 patients with refractory anemia 10
- Clinical haematology comparative 368 (B)
- Clinical hematology fundamentals (4th ed.) 63 (B)
- Clotting factors screening coagulation tests and clotting factors in homozygous β -thalassaemia 358
- Coagulation of blood, v Coagulation tests, Hämorrhagische Diathesen (B), Plasminogen activator Platelet aggregation, Thrombin (B), Thrombosis (B)
- Coagulation tests screening coagulation tests and clotting factors in homozygous β -thalassaemia 358
- Cobaltous chloride v CoCl₂
- CoCl treatment erythrocyte acetylcholin-

- enzyme activity changes in the early stages of anemic and hypobaric hypoxia or after CoCl_2 treatment in rabbits 21
- Colony formation: collection of *in vitro* colony-forming units from dogs by repeated continuous flow leukapheresis 172
- Common origin of lympho-myeloid complex in man, preliminary evidence 29
- Comparative clinical haematology 368 (B)
- Computer program: prognostic factors in multiple myeloma (A retrospective study using conventional statistical methods as a computer program) 57
- Congenital hypoplastic anemia refractory to corticosteroids but responding to cyclophosphamide and antilymphocytic globulin (Report of case having responded with transitory wave of dyserythropoiesis) 90
- Contact coagulation factors, Coagulation tests
- Cooley's anemia, Thalassemia
- Corticosteroids: congenital hypoplastic anemia, refractory to corticosteroids but responding to cyclophosphamide and antilymphocytic globulin (Report of case having responded with transitory wave of dyserythropoiesis) 90
- ^{51}Cr Platelet count
- Cryoglobulinemic neuropathy peripheral, in a patient with GAUCHER disease 117
- Cultures des cellules, Colony formation, Iron uptake by cell suspensions (fibroblasts, lymphoblasts)
- Cyclophosphamide: congenital hypoplastic anemia, refractory to corticosteroids but responding to cyclophosphamide and antilymphocytic globulin (Report of case having responded with transitory wave of dyserythropoiesis) 90
- Cytophotometry MAY-HIOURV anomaly
- Cytoplasmic immunoglobulins in bone marrow cells of polyclonal and of monoclonal origin 137
- Cytostatics, Benznaphen, Cyclophosphamide, Methotrexate
- Defect of bone marrow granulocyte reserve in liver cirrhosis evaluated with etiocholanolone 291
- Density of erythrocytes distribution: relationship of density distribution and pyruvate kinase electrophoretic pattern of erythrocytes in sickle cell diseases and other disorders 201
- Deutsche Gesellschaft für Hämatologie und Onkologie, 24. Jahrestagung, gemeinsam mit der Österreichischen Gesellschaft für Hämatologie und Onkologie (Göttingen, 8. 11. Oktober 1978) 128
- Diabetic neuropathy platelet aggregation 53
- Diagnostik hämorrhagischer Diathesen (Methodische Fortschritte im medizinischen Laboratorium, Bd. 4) 365 (B)
- Dialysis, acid dialysis, Folate binder
- DRUMMOND-BLACKMAN's anemia, v Hypoplastic anemia, congenital
- Diathesen, hämorrhagische, Diagnostik (Methodische Fortschritte im medizinischen Laboratorium, Bd. 4) 365 (B)
- Differentiation between benign and malignant monoclonal gammopathies by discriminant analysis on serum and bone marrow parameters 129
- Discriminant analysis on serum and bone marrow parameters: differentiation between benign and malignant monoclonal gammopathies by discriminant analysis on serum and bone marrow parameters 129
- Diseases: Erkrankungen der Myelopose 126 (B)
- infectious, hematology 128 (B)
- Disorders of lymphopoiesis and lymphoid function (Clinica in haematology Vol. 6) 62 (B)
- Dog: collection of *in vitro* colony-forming units from dogs by repeated continuous flow leukapheresis 172
- methotrexate and catrovirus factor after histoincompatible allogeneic bone marrow transplants in dog 233

- Blood group phenotypes and the origin of sickle cell hemoglobin in *Sicilians* 350
- Blood transfusion v Transfusion (B)
- Blutgruppen, v Blood group phenotypes
- Bluttransfusion, v Transfusion (B)
- Bone marrow cell suspensions, Iron uptake, v Iron uptake by cell suspensions
- Bone marrow cells cytoplasmic immunoglobulins in bone marrow cells of polyclonal and of monoclonal origin 137
- Bone marrow granulocyte reserve, defect in liver cirrhosis, evaluated with etiocholanolone 291
- Bone marrow parameters differentiation between benign and malignant monoclonal gammopathies by discriminant analysis on serum and bone marrow parameters 129
- Bone marrow transplants methotrexate and citrovorum factor after histoincompatible allogeneic bone marrow transplants in dog 233
- Book reviews 62-64 (B) 126-128 (B) 365-369 (B)
- B (T) lymphocytes, v Red cell aplasia (adult pure)
- B (T) rosetting lymphocytes, v Mouse red cell
- Buchbesprechungen 62-64 (B), 126-128 (B), 365-369 (B)
- Busulphan, v Megakaryocytic leukaemia
- Canis*, v Dog
- Canine leukocytes, v Colony formation (*in vitro*)
- ¹⁴C-5CH₃H₄pteroylglutamic acid, v Folate binder
- Cell cultures, v Colony formation, Iron uptake by cell suspensions (fibroblasts, lymphoblasts)
- Cell electrophoresis, v Platelet membrane
- Cell suspensions from bone marrow and liver v Iron uptake by cell suspensions
- Chain, α -chain interaction between iron deficiency and α -thalassaemia the *in vitro* effect of haemin on α -chain synthesis 1
- Chemistry and biology of thrombin 368(B)
- Chemotherapy v Non-Hodgkin's lymphoma
- Children platelet counts in children with iron deficiency anemia 81
- Children, *Turkish* children decreased iron and zinc absorption in *Turkish* children with iron deficiency and geophagia 76
- Chlorpromazine, v Red cell flexibility
- Chromatography v Folate binder Folic acid binding
- Chromosome, Philadelphia chromosome, v Lympho-myeloid complex
- Chromosomes, v Refractory anemia
- Chronic hemolysis, v Coagulation tests
- Chronic leukemias (Clinica in haematology Vol. 6 Nr 1) 64 (B)
- Cirrhosis of the liver defect of bone marrow granulocyte reserve in liver cirrhosis evaluated with etiocholanolone 291
- v Alcoholic cirrhosis
- Citrovorum factor and methotrexate after histoincompatible allogeneic bone marrow transplants in dogs 233
- 'Classic and 'acute myelofibrosis (A retrospective study) 103
- Clinical contribution to the knowledge of hemopoietic dysplasias long-term follow-up of 13 patients with refractory anemia 10
- Clinical haematology comparative 368(B)
- Clinical hematology fundamentals (4th ed.) 63 (B)
- Clotting factors screening coagulation tests and clotting factors in homozygous β -thalassaemia 358
- Coagulation of blood, v Coagulation tests, Hämorrhagische Diathesen (B), Plasminogen activator Platelet aggregation, Thrombin (B) Thrombosis (B)
- Coagulation tests screening coagulation tests and clotting factors in homozygous β -thalassaemia 358
- Cobaltous chloride, v CoCl₂
- CoCl₂ treatment erythrocyte acetylcholin-

- enzyme activity changes in the early stages of anemic and hypobaric hypoxia or after CoCl_2 treatment in rabbits 21
- Colony formation: collection of *in vivo* colony-forming units from dogs by repeated continuous flow leukapheresis 172
- Common origin of a lympho-myeloid complex in man, preliminary evidence 29
- Comparative clinical haematology 368 (B)
- Computer programs: prognostic factors in multiple myeloma (A retrospective study using conventional statistical methods as a computer program) 257
- Congenital hypoplastic anaemia refractory to corticosteroids but responding to cyclophosphamide and antilymphocytic globulin (Report of a case having responded with a transitory wave of dyserythropoiesis) 90
- Contact coagulation factors, Coagulation tests
- COOLLY' anaemia, Thalassemia
- Corticosteroids: congenital hypoplastic anaemia, refractory to corticosteroids but responding to cyclophosphamide and antilymphocytic globulin (Report of a case having responded with a transitory wave of dyserythropoiesis) 90
- ^{51}Cr Platelet count
- Cryoglobulinemic neuropathy peripheral, in patient with GAUCHER'S disease 117
- Cultures des cellules, Colony formation, Iron uptake by cell suspensions (fibroblasts, lymphoblasts)
- Cyclophosphamide: congenital hypoplastic anaemia, refractory to corticosteroids but responding to cyclophosphamide and antilymphocytic globulin (Report of a case having responded with a transitory wave of dyserythropoiesis) 90
- Cytophotometry MA HROULYANOMY
- Cytoplasmic immunoglobulins in bone marrow cells of polyclonal and of monoclonal origin 157
- Cytostatics, Busulphan, Cyclophosphamide, Methotrexate
- Defect of bone marrow granulocyte reserve in liver cirrhosis evaluated with eriocholalone 291
- Density of erythrocytes distribution: relationship of density distribution and pyruvate kinase electrophoretic pattern of erythrocytes in sickle cell diseases and other disorders 201
- Deutsche Gesellschaft für Hämatologie und Onkologie 24. Jahrestagung, gemeinsam mit der Österreichischen Gesellschaft für Hämatologie und Onkologie (Öttingen, 8. 11. Oktober 1978) 128
- Diabetic retinopathy: platelet aggregation 53
- Diagnostik hämorrhagischer Diathesen (Methodische Fortschritte im medizinischen Laboratorium, Bd. 4) 365 (B)
- Dialysis, acid dialysis, Foies bides
- DIAMOND-BLACKFAN'S anaemia, v Hypoplastic anaemia, congenital
- Diathesen, hämorrhagische, Diagnostik (Methodische Fortschritte im medizinischen Laboratorium, Bd. 4) 365 (B)
- Differentiation between benign and malignant monoclonal gammopathies by discriminant analysis on serum and bone marrow parameters 129
- Discriminant analysis on serum and bone marrow parameters: differentiation between benign and malignant monoclonal gammopathies by discriminant analysis on serum and bone marrow parameters 129
- Disorders Eritrimologien der Myeloposen 126 (B)
- infections, hematology 128 (B)
- Disorders of lymphopoiesis and lymphoid function (Clinics in haematology Vol. 6) 62 (B)
- Dog: collection of *in vivo* colony-forming units from dogs by repeated continuous flow leukapheresis 172
- methotrexate and cloveracin factor after histoincompatible allogeneic bone marrow transplants in dog 233

- Blood group phenotypes and the origin of sickle cell hemoglobin in *Stellans* 350
- Blood transfusion v Transfusion (B)
- Blutgruppen, v Blood group phenotypes
- Bluttransfusion, v Transfusion (B)
- Bone marrow cell suspensions, iron uptake, v Iron uptake by cell suspensions
- Bone marrow cells cytoplasmic immunoglobulins in bone marrow cells of polyclonal and of monoclonal origin 137
- Bone marrow granulocyte reserve, defect in liver cirrhosis, evaluated with etiocholanolone 291
- Bone marrow parameters differentiation between benign and malignant monoclonal gammopathies by discriminant analysis on serum and bone marrow parameters 129
- Bone marrow transplants methotrexate and citrovorum factor after histoincompatible allogeneic bone marrow transplants in dog 233
- Book reviews 62-64 (B), 126-128 (B) 365-369 (B)
- B (T) lymphocytes, v Red cell aplasia (adult, pure)
- B (T) rosetting lymphocytes, v Mouse red cell
- Buchbesprechungen 62-64 (B) 126-128 (B), 365-369 (B)
- Busulphan, v Megakaryocytic leukaemia
- Canis*, v Dog
- Canine leukocytes, v Colony formation (*in vitro*)
- ^4C -5CH₃H₄pteroylglutamic acid, v Folate binder
- Cell cultures, v Colony formation, Iron uptake by cell suspensions (fibroblasts, lymphoblasts)
- Cell electrophoresis, v Platelet membrane
- Cell suspensions from bone marrow and liver v Iron uptake by cell suspensions
- Chain, α -chain Interaction between iron deficiency and α -thalassaemia the *in vitro* effect of haemin on α -chain synthesis 1
- Chemistry and biology of thrombin 368 (B)
- Chemotherapy v Non-HODGKIN's lymphoma
- Children platelet counts in children with iron deficiency anemia ■
- Children, *Turkish* children decreased iron and zinc absorption in *Turkish* children with iron deficiency and geophagia 76
- Chlorpromazine, v Red cell flexibility
- Chromatography v Folate binder Folate acid binding
- Chromosome, Philadelphia chromosome, v Lympho-myeloid complex
- Chromosomes, v Refractory anemia
- Chronic hemolysis, v Coagulation tests
- Chronic leukemias (Clinics in haematology Vol. 6, Nr 1) 64 (B)
- Cirrhosis of the liver defect of bone marrow granulocyte reserve in liver cirrhosis evaluated with etiocholanolone 291
- v Alcoholic cirrhosis
- Citrovorum factor and methotrexate after histoincompatible allogeneic bone marrow transplants in dogs 233
- 'Classic and acute myelofibrosis (A retrospective study) 108
- Clinical contribution to the knowledge of hemopoietic dysplasias long-term follow-up of 13 patients with refractory anemia 10
- Clinical haematology comparative 368 (B)
- Clinical hematology fundamentals (4th ed.) 63 (B)
- Clotting factors screening coagulation tests and clotting factors in homozygous β -thalassaemia 358
- Coagulation of blood, v Coagulation tests, Hämorrhagische Diathesen (B), Plasminogen activator Platelet aggregation, Thrombin (B), Thrombosis (B)
- Coagulation tests screening coagulation tests and clotting factors in homozygous β -thalassaemia 358
- Cobaltous chloride, v CoCl₂
- CoCl₂ treatment erythrocyte acetylcholin

- Functional heterogeneity of transferrin-bound iron (Iron uptake by cell suspensions from bone marrow and liver and by cell cultures of fibroblasts and lymphoblasts) 210
- Fundamentals of clinical hematology (4th ed.) 63 (B)
- Gammopathies differentiation between benign and malignant monoclonal gammopathies by discriminant analysis on serum and bone marrow parameters 129
- Cytoplasmic immunoglobulins
- Gautschi's disease peripheral cryoglobulinemic neuropathy in a patient with GAUTSCHI'S disease 117
- Genetics, v Leukaemia, lymphocytic, chronic (In Larue)
- Geophagia decreased iron and zinc absorption in Turkish children with iron deficiency and geophagia 76
- Gesellschaften (Tagungen), Hämatologie
- Globin synthesis, Alpha-chain
- Globulin, antilymphocytic, Antilymphocytic globulin
- Globulins, v Cryoglobulinemic neuropathy Gammopathies
- Graft-versus-host disease, Allogeneic bone marrow transplantation
- Granulocyte reserve of the bone marrow defect, in liver cirrhosis, evaluated with ethocholanolins 291
- Granulocytic leukaemia, chronic folic acid binding protein in chronic granulocytic leukaemia the effect of methotrexate 341
- Granulocytic leukemia, chronic, neutrophilic type, with paraproteinemia (IgA type K) 316
- GRUPPE, RUTTE
- Grupos sanguíneos, Blood group phenotypes
- Growth kinetics of tumours 369 (B)
- Hämatologie Deutsche Gesellschaft für Hämatologie und Onkologie, 24. Jahrestagung, gemeinsam mit der Österreichischen Gesellschaft für Hämatologie und Onkologie (Göttingen, 8. 11. Oktober 1978) 128
- Hematology clinical, fundamentals (4th ed.) 63 (B)
- Hematology comparative, clinical 368 (B)
- Hematology examination 127 (B)
- Hematology of infectious diseases 128 (B)
- Hematology practical (5th ed.) 366 (B)
- Hematology trends 63 (B)
- Hematology v Hämatologie
- Haemlin and protoporphyrin, binding to human spectrin 321
- Haematochromatosis, idiopathic influence of food iron absorption on the plasma iron level in idiopathic haematochromatosis 65
- Hämmodulation Die akute normovolumische Hämmodulation in klinischer Anwendung (Anesthesiology and Resuscitation, Vol. 10-5) 64 (B)
- Haemoglobin, sickle cell haemoglobin, blood group phenotypes and the origin of sickle cell haemoglobin (in Stiller) 350
- Haemoglobin S, v Haemoglobin, sickle cell haemoglobin, Sickle cell diseases
- Haemoglobin synthesis, regulation 127 (B)
- Beta-thalassaemia
- Haemoglobin, Alpha-chain, Haemlin
- Haemoglobinopathies (Major problems in internal medicine, Vol. XII) 127 (B)
- Haemoglobinopathies, Haemoglobin, sickle cell haemoglobin Sickle cell diseases Thalassemia
- Haemoglobins, human 367 (B)
- Haemolysis, chronic, Coagulation tests.
- Haemolytic anaemia, Leukaemia, lymphocytic, chronic (In Larue)
- Haemopoietic dysplasias clinical contribution to the knowledge of haemopoietic dysplasias long-term follow-up of 13 patients with refractory anaemia 10
- Hämothologische Dathesen, Diagnostik (Methodische Fortschritte im medizinischen Laboratorium, Bd. 4) 365 (B)
- Hb S, Haemoglobin S

- Duodenal biopsy v Iron and zinc absorption
- Dyserythropoiesis congenital hypoplastic anaemia, refractory to corticosteroids but responding to cyclophosphamide and antilymphocytic globulin (Report of a case having responded with a transitory wave of dyserythropoiesis) 90
- v Refractory anaemia
- Eisen, v Iron deficiency
- Electrokinetic charge, v Platelet membrane
- Electrophoresis, v Benicz Jones proteinuria, Pyruvate kinase electrophoresis
- Electrophoresis, cell electrophoresis, v Platelet membrane
- Enfants, v Children
- Epinephrine, v Platelet aggregation
- Erkrankungen der Myelopoese 126 (B)
- Erythrocyte acetylcholinesterase activity changes in the early stages of anemic and hypobaric hypoxia or after CoCl₂ treatment in rabbits 21
- Erythrocyte flexibility in spherocytosis, studies, using a polycarbonate membrane filtration method 329
- Erythrocyte aplasia (adult, pure) para proteinemic variety of pure red cell aplasia (Immunological studies in 1 patient) 227
- Erythrocytes, density distribution relationship of density distribution and pyruvate kinase electrophoretic pattern of erythrocytes in sickle cell diseases and other disorders 201
- Erythrocytes membrane transport in red cells 366 (B)
- Erythrocytes of mouse significance of mouse red cell rosette-forming lymphocytes in chronic lymphocytic leukaemia 36
- Erythrocytes, sedimentation rate, v Myelofibrosis
- Erythropoiesis, IgG inhibitor paraproteinemic variety of pure red cell aplasia (Immunological studies in 1 patient) 227
- Erythropoiesis, v Dyserythropoiesis
- Esterase, kallikrein esterase, v Coagulation tests
- Ethnical and clinical aspects of chronic lymphocytic leukemia in Israel (A survey on 288 patients) 161
- Etiocolanalone test defect of bone marrow granulocyte reserve in liver cirrhosis, evaluated with etiocolanalone 291
- Evolution of haemopoietic dysplasias, v Haemopoietic dysplasias
- Examination haematology 127 (B)
- Factor XI (= Plasma prothrombin antecedent = PTA), v Coagulation tests
- Factor XII (= Hagemann factor), v Coagulation tests
- Family v Leukaemia, lymphocytic, chronic (in Israel)
- Fatty acids, polyunsaturated, v Serum lipid
- ⁵⁵Fe, v Inorganic iron absorption, iron uptake
- Fer v Iron deficiency
- Ferments, v Acetylcholinesterase, Pyruvate kinase electrophoresis, Urokinase (B)
- Ferritin, v Plasma iron level
- Fibrinogen, v Plasminogen activator
- Fibrinolytic system, v Plasminogen activator
- Fibroblasts, cell cultures, iron uptake, v Iron uptake by cell suspensions
- Filtration method, v Red cell flexibility
- Flexibility of red cells in spherocytosis, studies, using a polycarbonate membrane filtration method 329
- Fole, v Liver
- Folate binder comparison between the unsaturated plasma folate binder and *in vivo* labelled plasma folate binder 296
- Folic acid binding protein in chronic granulocytic leukaemia the effect of methotrexate 341
- Food iron absorption, influence on the plasma iron level in idiopathic hemochromatosis 63
- Fortschritte, v Hämorrhagische Diathesen (B)

- Functional heterogeneity of transferrin-bound iron (Iron uptake by cell suspensions from bone marrow and liver and by cell cultures of fibroblasts and lymphoblasts) 210
- Fundamentals of clinical hematology (4th ed.) 63 (B)
- Gammopathies differentiation between benign and malignant monoclonal gammopathies by discriminant analysis on serum and bone marrow parameters 129
- Cytoplasmic immunoglobulins
- GADGNER's disease peripheral cryoglobulinemic neuropathy in a patient with GADGNER's disease 117
- Genetics, v Leukaemia, lymphocytic, chronic (In brief)
- Geophagia decreased iron and zinc absorption in Turkish children with iron deficiency and geophagia 76
- Gesellschaften (Tagungen), v Hämatologie
- Globin synthesis, v Alpha-chain
- Globulin, antilymphocytic, Antilymphocytic globulin
- Globulins, Cryoglobulinemic neuropathy Gammopathies
- Graft-versus-host disease, v Allogeneic bone marrow transplantation
- Granulocyte reserve of the bone marrow defect, in liver cirrhosis, evaluated with ethochololones 291
- Granulocytic leukaemia, chronic: folic acid binding protein in chronic granulocytic leukaemia the effect of methotrexate 341
- Granulocytic leukaemia, chronic, neutrophilic type, with paraproteinemia (IgA type K) 316
- GASTR, v KUTR
- Groupes sanguins, Blood group phenotypes
- Growth kinetics of tumours 369 (B)
- Hämatologie Deutsche Gesellschaft für Hämatologie und Onkologie, 24. Jahrestagung, gemeinsam mit der Österreichischen Gesellschaft für Hämatologie und Onkologie (Göttingen, 8.-11. Oktober 1978) 128
- Haematology clinical, fundamentals (4th ed.) 63 (B)
- Haematology comparative, clinical 368 (B)
- Haematology examination 127 (B)
- Haematology of infectious diseases 128 (B)
- Haematology practical (5th ed.) 366 (B)
- Haematology trends III (B)
- Haematologie Hämatologie
- Haemin and protoporphyrin, binding to human spectra 321
- Haematochromatosis, idiopathic influence of food iron absorption on the plasma iron level in idiopathic haematochromatosis 61
- Hämofiltration Die akute normovolämische Hämofiltration in klinischer Anwendung (Anaesthesiology and Resuscitation, Vol. 104) 64 (B)
- Haemoglobin, sickle cell haemoglobin blood group phenotypes and the origin of sickle cell haemoglobin in Silesia 350
- Haemoglobin E, Haemoglobin, sickle cell haemoglobin, Sickle cell diseases
- Haemoglobin synthesis, regulation 127 (B)
- Beta-thalassaemia
- Haemoglobin, Alpha-chain, Haemin
- Haemoglobinopathies (Major problems in internal medicine, Vol. XII) 127 (B)
- Haemoglobinopathies, Haemoglobin, sickle cell haemoglobin Sickle cell diseases Thalassemia
- Haemoglobins, human 367 (B)
- Haemolysis, chronic, Coagulation tests
- Haemolytic anaemia, Leukaemia, lymphocytic, chronic (In brief)
- Haemopoietic dysplasias clinical contribution to the knowledge of haemopoietic dysplasias long-term follow-up of 13 patients with refractory anaemia 10
- Hämorrhagische Diathesen, Diagnostik (Methodische Fortschritte im medizinischen Laboratorium, Bd. 4) 365 (B)
- Hb E, Haemoglobin E

HEGGLIN, v MAY

Hematology clinical fundamentals (4th ed.) 63 (B)

Hematology comparative clinical hematology 368 (B)

Hematology examination 177 (B)

Hematology of infectious diseases 128 (B)

Hematology practical (5th ed.) 366 (B)

Hematology trends 63 (B)

Hematology v Hämatologie, Haematology

Hemochromatosis, idopathic Influence of food iron absorption on the plasma iron level in idopathic hemochromatosis 65

Hemoglobin, sickle cell hemoglobin blood group phenotypes and the origin of sickle cell hemoglobin in *Sicilians* 350

Hemoglobin synthesis, v Beta-thalassemia

Hemoglobinopathies (Major problems in internal medicine, Vol XII) 127 (B)

Hemoglobins, human 367 (B)

Hemolysis, chronic v Coagulation tests

Hemolytic anemia v Leukemia, lymphocytic, chronic (in *Israel*)

Hemopoietic dysplasias clinical contribution to the knowledge of hemopoietic dysplasias long-term follow-up of 13 patients with refractory anemia 10

Hem -, v Haem -, Häm

Hepar v Liver

Heredopathies, v (Gammopathies) Haemoglobinopathies Hereditary (Hypoplastic anemia, congenital)

Hereditary spherocytosis, v Erythrocytes, density distribution

Heterogeneity functional of transferrin-bound iron (Iron uptake by cell suspensions from bone marrow and liver and by cell cultures of fibroblasts and lymphoblasts) 210

³H folic acid v Folic acid binding

Histiocytic lymphoma, v Non-Hodgkin's lymphoma

Histo-incompatibility methotrexate and citrovorum factor after histoincompatible allogeneic bone marrow transplants in dog 233

³H-methotrexate v Methotrexate

Hodgkin, morbus Hodgkin, v Non-Hodgkin's lymphoma

Homozygous β -thalassemia screening coagulation tests and clotting factors in homozygous β -thalassemia 358

³H-pteroylglutamic acid, v Folate binder

³H-thymidine, v Lymphocyte transformation tests

Human hemoglobins 367 (B)

Human platelet membrane, amino groups 304

Human spectrin binding of protoporphyrin and haemin to human spectrin 321

Human -, v Man

Hypobaric and anemic hypoxia erythrocyte acetylcholinesterase activity changes in the early stages of anemic and hypobaric hypoxia or after CoCl_2 treatment in rabbits 21

Hypoplastic anaemia, congenital, refractory to corticosteroids but responding to cyclophosphamide and antilymphocytic globulin (Report of a case having responded with a transitory wave of dyserythropoiesis) 90

Hypoxia, anemic and hypobaric erythrocyte acetylcholinesterase activity changes in the early stages of anemic and hypobaric hypoxia or after CoCl_2 treatment in rabbits 21

¹²⁵I-fibrin v Plasminogen activator

⁵⁹Fe-transferrin, v Iron uptake

Idopathic BENCK-JONAS proteinuria 269

Idopathic hemochromatosis Influence of food iron absorption on the plasma iron level in idopathic hemochromatosis 65

IgA type K, chronic granulocytic leukemia neutrophilic type, with paraproteinemia (Ig A type K.) 316

IgA, IgG IgM v Cytoplasmic immunoglobulins

IgG serum inhibitor of erythropoiesis paraproteinemic variety of pure red cell aplasia (Immunological studies in patient) 227

- IgM-bearing lymphocytes, Red cell aplasia (adult, pure)
- Immunochemical analysis, Bruce Jones proteinuria
- Immunofluorescence, v Lymphoid antigens
- Immunoglobulins, cytoplasmic, in bone marrow cells of polyclonal and of monoclonal origin 137
- Immunoglobulins, v Gammapathies, Leukemia, lymphocytic, chronic (in Israel)
- Immunological disorder, v Red cell aplasia (adult, pure)
- Immunosuppression, v Allogeneic bone marrow transplantation, Corticosteroids
- Infectious diseases, herpetology 128 (B)
- Inorganic iron absorption in subjects with iron deficiency anemia, achylia gastrica and alcoholic cirrhosis using whole-body counter 182
- Interaction between iron deficiency and α -thalassaemia: the *in vitro* effect of haem on α -chain synthesis 1
- Interaction of haemin and spectrin, Haemin
- Intrathecal methotrexate causing paraplegia in a middle-aged woman 59
- In vitro* colony-forming units from dogs, collection, by repeated continuous flow leukapheresis 172
- Iron, absorption: influence of food iron absorption on the plasma iron level in idiopathic hemochromatosis 65 ; inorganic iron absorption in subjects with iron deficiency anemia, achylia gastrica and alcoholic cirrhosis using a whole-body counter 182
- Iron deficiency: interaction between iron deficiency and α -thalassaemia: the *in vitro* effects of haem on α -chain synthesis 1
- Iron deficiency anemia in children, platelet counts 85
- Iron excess, Aberration of iron and porphyrin metabolism 349 (B)
- Iron metabolism, Iron uptake.
- Iron, transferrin-bound, functional heterogeneity (Iron uptake by cell suspensions from bone marrow and liver and by cell cultures of fibroblasts and lymphoblasts) 210
- Iron uptake by cell suspensions from bone marrow and liver and by cell cultures of fibroblasts and lymphoblasts. Functional heterogeneity of transferrin-bound iron 210
- Iron and zinc absorption, decreased, in Turkish children with iron deficiency and geophagia 76
- Isotopes, radioactive, v Folate binder, Folic acid binding, Inorganic iron absorption, Iron uptake, Lymphocyte transformation tests, Platelet count
- Israel: ethnical and clinical aspects of chronic lymphocytic leukemia in Israel (A survey on 288 patients) 161
- Jews in Israel from East Europe from Asia and Africa or born in Israel, Lymphocytic leukemia, chronic
- Kallikrein esterase, v Coagulation tests
- Kinder, Children
- Klinisch-diagnostisches Laboratorium (Methoden), Hämorrhagische Diathesen (B)
- Klinisch, v Clinical
- Knochenmark, v Bone marrow
- Kobalchlorid, CoCl_2
- Kongenital, Congenital ..
- Kryoglobulinämie, Cryoglobulinemic neuropathy
- Laboratorium, Methoden, Hämorrhagische Diathesen (B)
- Lactic dehydrogenase (serum), v Myofibrils
- Leber, Liver
- Lehrplan des Transfusionswesens (Schriftreihe d. Akademie f. Ärztliche Fortbildung d. DDR, Bd. 45) 347 (B)
- Leukaemia, granulocytic, chronic: folic acid binding protein in chronic granulo-

HEODLIN, v MAY

Hematology clinical, fundamentals (4th ed.) 63 (B)

Hematology comparative clinical hematology 363 (B)

Hematology examination 127 (B)

Hematology of infectious diseases 128 (B)

Hematology practical (5th ed.) 366 (B)

Hematology trends III (B)

Hematology v Hämatologie, Haematology

Hemochromatosis, idiopathic Influence of food iron absorption on the plasma iron level in idiopathic hemochromatosis 65

Hemoglobin, sickle cell hemoglobin blood group phenotypes and the origin of sickle cell hemoglobin in *Sicilians* 350

Hemoglobin synthesis, v Beta-thalassemia

Hemoglobinopathies (Major problems in Internal medicine, Vol. XII) 127 (B)

Hemoglobins, human 367 (B)

Hemolysis, chronic, v Coagulation tests

Hemolytic anemia, v Leukemia, lymphocyte, chronic (in *Israel*)

Hemopoietic dysplasias clinical contribution to the knowledge of hemopoietic dysplasias long-term follow-up of 13 patients with refractory anemia 10

Hem v Haem -, Häm

Hepar v Liver

Heredopathies, v (Gammopathies) Haemoglobinopathies Hereditary (Hypoplastic anemia, congenital)

Hereditary spherocytosis, v Erythrocytes, density distribution

Heterogeneity functional, of transferrin-bound iron (Iron uptake by cell suspensions from bone marrow and liver and by cell cultures of fibroblasts and lymphoblasts) 210

³H folic acid v Folic acid binding

Histiocytic lymphoma, v Non-Hodgkin's lymphoma

Histoincompatibility methotrexate and citrovorum factor after histoincompatible allogeneic bone marrow transplants in dog 233

³H-methotrexate, v Methotrexate

HODGKIN morbus HODGKIN, v Non-HODGKIN's lymphoma

Homozygous β -thalassemia screening coagulation tests and clotting factors in homozygous β -thalassemia 358

³H-pteroylglutamic acid, v Folate binder

³H-thymidine, v Lymphocyte transformation tests

Human hemoglobins 367 (B)

Human platelet membrane, amino groups 304

Human spectrin binding of protoporphyrin and haemin to human spectrin 321

Human -, v Man

Hypobaric and anemic hypoxia erythrocyte acetylcholinesterase activity changes in the early stages of anemic and hypobaric hypoxia or after CoCl₂ treatment in rabbits 21

Hypoplastic anaemia, congenital, refractory to corticosteroids but responding to cyclophosphamide and antilymphocytic globulin (Report of a case having responded with a transitory wave of dyserythropoiesis) 90

Hypoxia, anemic and hypobaric erythrocyte acetylcholinesterase activity changes in the early stages of anemic and hypobaric hypoxia or after CoCl₂ treatment in rabbits 21

¹²⁵I-fibrin, v Plasminogen activator

⁵⁹Fe-transferrin, v Iron uptake

Idiopathic BENCKE JONES proteinuria 269

Idiopathic hemochromatosis Influence of food iron absorption on the plasma iron level in idiopathic hemochromatosis 65

IgA type K, chronic granulocytic leukemia, neutrophilic type, with paraproteinemia (Ig A type K) 316

IgA IgG IgM v Cytoplasmic immunoglobulins

IgG serum inhibitor of erythropoiesis paraproteinemic variety of pure red cell aplasia (Immunological studies in 1 patient) 227

- ethical and clinical aspects (A survey on 211 patients) 161
- Lympho-hemopoietic stem cells, Lympho-myeloid complex
- Lymphoid antigens on blast cells in the agranular metamorphosis of chronic myelogenous leukaemia 280
- Lymphoid blastic crisis, v Lymphoid antigens
- Lymphoid function Disorders of lymphopoiesis and lymphoid function (Clinics in haematology Vol. 6) 62 (B)
- Lymphoid leukaemia, acute chronic myeloid leukaemia initiating as acute lymphoid leukaemia 56
- Lymphoma, non-Hodgkin's lymphoma terminating in acute myelogenous leukaemia 250
- Lympho-myeloid complex preliminary evidence of the common origin of lympho-myeloid complex in man 29
- Lymphopoiesis disorders of lymphopoiesis and lymphoid function (Clinics in haematology Vol. 6) 62 (B)
- Malignant gammopathies, v Gammopathies
- Man preliminary evidence for the common origin of lympho-myeloid complex in man 29
- Human
- Mars, v Moore
- MAY-HIOGLIN anomaly megakaryocyte polyploidization 45
- M cells (mouse red cell rosetting lymphocytes), Mouse red cell
- M component, serum, Red cell aplasia (adult, pure)
- M components, 'idiopathic' Bence Jones proteinuria
- Mediterranean anaemia, Thalassemia
- Medulla ossea, v Bone marrow
- Megakaryocyte polyploidization in MA HIOGLIN anomaly 45
- Megakaryocytic leukaemia (A case of unusual outcome) 122
- Membrane filtration method (polycarbonate membrane), v Red cell flexibility
- Membrane of platelets study of amino groups of the human platelet membrane 304
- Membrane transport in red cells 366 (B)
- Meningeal leukaemia, v Methotrexate, intrathecal
- Metabolism of iron and porphyrin, aberrations, Iron excess 369 (B)
- Metamorphosis, agranular of chronic myelogenous leukaemia, lymphoid antigens on blast cells 280
- Method new method for the detection of the plasminogen activator content of vein walls 243
- studies on red cell flexibility in spherocytosis, using a polycarbonate membrane filtration method 329
- Methoden, v Hämorrhagische Diathesen (B)
- Methotrexate and citrovorum factor after histocompatible allogeneic bone marrow transplants in dogs 233
- Methotrexate, intrathecal, causing paraplegia in middle-age woman 59
- Methotrexate (TH-methotrexate) folic acid binding protein in chronic granulocytic leukaemia the effect of methotrexate 341
- Metoprolol, Platelet count
- Mice, v Mouse
- Microgli, Remy-
- Microautoradiography v Autoradiography
- Minio osseae, Bone marrow
- Monoclonal gammopathies differentiation between benign and malignant monoclonal gammopathies by discriminant analysis on serum and bone marrow parameters 129
- Monoclonal plasma cells of bone marrow Cytoplasmic immunoglobulins
- Mouse red cell rosette-forming lymphocytes in chronic lymphocytic leukaemia, significance 36
- Multiple myeloma prognostic factors (A retrospective study using conventional

- cytic leukaemia the effect of methotrexate 341
- neutrophilic type, with paraproteinemia (IgA type K) 316
- Leukaemia, lymphatic, chronic, v Mouse red cell
- Leukaemia, lymphocytic, chronic ethical and clinical aspects of chronic lymphocytic leukaemia in *Israel* (A survey on 288 patients) 161
- Leukaemia, lymphoid, acute chronic myeloid leukaemia initiating as acute lymphoid leukaemia 56
- Leukaemia, megakaryocytic (A case of unusual outcome) 122
- Leukaemia, myelogenous, acute non-Hodgkin's lymphoma terminating in acute myelogenous leukaemia 50
- Leukaemia, myelogenous, chronic lymphoid antigens on blast cells in the agranular metamorphosis of chronic myelogenous leukaemia 280
- Leukaemia, myeloid, chronic initiating as acute lymphoid leukaemia 56
- v Lympho-myeloid complex
- Leukaemia surface antigen, v Lymphoid antigens
- Leukaemia, v Preleukemia
- Leukaemias, chronic (Clinica in haematology Vol. 6, Nr 1) 64 (B)
- Leukapheresis collection of *in vitro* colony-forming units from dogs by repeated continuous flow leukapheresis 172
- Leukemia, granulocytic, chronic, neutrophilic type, with paraproteinemia (IgA type K) 316
- Leukemia, lymphocytic, chronic ethical and clinical aspects of chronic lymphocytic leukemia in *Israel* (A survey on 288 patients) 161
- Leukemia, myelogenous, acute non-Hodgkin's lymphoma terminating in acute myelogenous leukemia 250
- Leukemia, myeloid, chronic, v Lympho-myeloid complex
- Leukemia, v Preleukemia
- Leukemias, chronic (Clinica in haematology Vol. 6, Nr 1) 64 (B)
- Leukem , v Leukaem
- Leukocytaires und retikuläres System I (Handb. d. inneren Medizin, Bd. 2, Teil 3) 126 (B)
- Leukocytes collection of *in vitro* colony-forming units from dogs by repeated continuous flow leukapheresis 172
- Leukocytes, supernatant (chronic granulocytic leukaemia), v Folic acid binding
- Liber 62-64 (B), 126-128 (B), 365-369 (B)
- Lipids serum lipid pattern in β -thalassaemia 100
- Liver cell suspensions, iron uptake, v Iron uptake by cell suspensions
- Liver cirrhosis defect of bone marrow granulocyte reserve in liver cirrhosis evaluated with etiocholanolone 291
- v Alcoholic cirrhosis
- Liver parenchymal cells, v Liver cell suspensions
- Livres nouveaux 62-64 (B), 126-128 (B), 365-369 (B)
- Lymphatic leukaemia, chronic, v Mouse red cell
- Lymphoblasts, cell cultures, iron uptake, v Iron uptake by cell suspensions
- Lymphocyte blastogenesis, v Lymphocyte transformation tests
- Lymphocyte markers, v Red cell aplasia (adult, pure)
- Lymphocyte stimulation evaluation, indices, v Lymphocyte transformation tests
- Lymphocyte transformation tests, multiple, by phytohemagglutinin in healthy individuals (Transient episodes of decreased lymphocyte blastogenesis) 148
- Lymphocytes, v Antilymphocytic globulin
- Lymphocytes, B and T lymphocytes, v Mouse red cell
- Lymphocytes, IgM bearing, v Red cell aplasia (adult, pure)
- Lymphocytes, M cells, v Mouse red cell
- Lymphocytic leukemia, chronic, in *Israel*

- the detection of the plasminogen activator content of vein walls 43
- Plasmocytosis in bone marrow aspirate, Bence Jones proteinuria
- Platelet aggregation in diabetic retinopathy 53
- Platelet count, peripheral platelet count in response to salbutamol before and after adrenergic beta-receptor blockade 310
- Platelets counts in children with iron deficiency anemia 85
- Platelet membrane, human, amino groups 304
- Polycarbonate membrane filtration studies on red cell flexibility in spherocytosis, using a polycarbonate membrane filtration method 329
- Polyclonal plasma cells of bone marrow Cytoplasmic immunoglobulins
- Polyploidization megakaryocyte polyploidization in MA -Fibrosis anomaly 45
- Polyunsaturated fatty acids, v Serum lipid
- Porphyrin and iron metabolism, aberrations. Iron excess 369 (B)
- Porphyrin, Protoporphyrin
- Practical haematology (5th ed) 366 (B)
- Proleukemia, Refractory anemia
- Prognosis of myelofibrosis, Myelofibrosis
- Prognostic factors in multiple myeloma (A retrospective study using conventional statistical methods and computer program) 257
- Progress, Hämorrhagische Diathesen (B)
- Propanolol, Platelet count
- Protein, binding folate acid binding protein in chronic granulocytic leukaemia the effect of methotrexate 341
- Protein (cytoplasmic surface of the erythrocyte membrane), Spectrin
- Proteinuria, 'idiopathic' Bence Jones proteinuria 269
- Protoporphyrin and haemia, binding to human spectrin 321
- PTA (= Plasma prothrombin antecedent factor = Factor XII), v Coagulation tests
- Pure red cell aplasia in the adult, v Red cell aplasia (adult, pure)
- Pyruvate kinase electrophoresis relationship of density distribution and pyruvate kinase electrophoretic pattern of erythrocytes in sickle cell diseases and other disorders 201
- Rabbit erythrocyte acetylcholinesterase activity changes in the early stages of anemic and hypobaric hypoxia or after CoCl_2 treatment in rabbits 11
- Radio-carbon, v Folate binder
- Radio-chrome, v Platelet count
- Radio-iodine, Iron uptake, Plasminogen activator
- Radio-iron, Inorganic iron absorption, Iron uptake, Plasminogen activator
- Receptor v Beta-receptor blockade
- Red cell aplasia (adult, pure) paraproteinaemic variety of pure red cell aplasia (Immunological studies in 1 patient) 277
- Red cell density distribution, Erythrocytes, density distribution
- Red cell flexibility in spherocytosis, studies, using polycarbonate membrane filtration method 329
- Red cells, Erythrocyte(s)
- Red cells, membrane transport 366 (B)
- Red cells of mouse, v Mouse red cell
- Refractory anemia clinical contribution to the knowledge of hemopoietic dysplasias, long-term follow-up of 111 patients with refractory anemia 10
- Regulation of haemoglobin synthesis 127 (B)
- Remission, Megakaryocytic leukaemia
- Reticulocytes, Myelofibrosis
- Ratuliferes and lymphocytes System L (Handb. d. Immun. Medizin, Bd. 2, Teil 3) 126 (B)
- Retinopathy diabetic, platelet aggregation 53
- Roth-Graeff-Mitchell disease, Beta-thalassemia

- statistical methods on a computer program) 257
- v Monoclonal gammopathies
- Mus musculus*, v Mouse
- Myelofibrosis, classic and 'acute (A retrospective study) 103
- Myelogenous leukaemia, chronic lymphoid antigens on blast cells in the agranular metamorphosis of chronic myelogenous leukaemia 280
- Myelogenous leukemia, acute non-Hodgkin's lymphoma terminating in acute myelogenous leukemia 250
- Myeloid leukaemia chronic, initiating as acute lymphoid leukaemia 56
- Myeloma, multiple prognostic factors (A retrospective study using conventional statistical methods and a computer program) 257
- v Monoclonal gammopathies
- Myelopoiesis, Erkrankungen 126 (B)
- Neoplasmas, multiple, primary v Leukaemia, lymphocytic, chronic (in Israel)
- Neuropathy cryoglobulinemic, peripheral, in a patient with GAUCHIER's disease 117
- Non-Hodgkin's lymphoma terminating in acute myelogenous leukemia 50
- Normovolämische Hämodilution Die akute normovolämische Hämodilution in klinischer Anwendung (Anaesthesiology and Resuscitation, Vol. 104) 64 (B)
- Österreichische Gesellschaft für Hämatologie und Onkologie, Tagung, gemeinsam mit der Deutschen Gesellschaft für Hämatologie und Onkologie (24. Jahrestagung) (Göttingen, 8.-11. Oktober 1978) 128
- Onkologie Deutsche Gesellschaft für Hämatologie und Onkologie, 24. Jahrestagung, gemeinsam mit der Österreichischen Gesellschaft für Hämatologie und Onkologie (Göttingen, 8.-11. Oktober 1978) 128
- Origin, common of a lympho-myeloid complex in man, preliminary evidence 29
- Paraplegia Intrathecal methotrexate causing paraplegia in a middle-age woman 59
- Paraproteinemia chronic granulocytic leukemia, neutrophilic type, with paraproteinemia (IgA type K) 316
- Paraproteinemic variety of pure red cell aplasia (Immunological study in 1 patient) 227
- Peripheral cryoglobulinemic neuropathy in a patient with GAUCHIER's disease 117
- Peripheral platelet count in response to salbutamol before and after adrenergic beta-receptor blockade 310
- Peroxidation, v Serum lipid
- Phenotypen, v Phenotypes of blood groups
- PHA, v Phytohemagglutinin
- Phenotypes of blood groups blood group phenotypes and the origin of sickle cell hemoglobin in *Stellans* 350
- Philadelphia chromosome, v Lympho-myeloid complex
- Ph-positive chronic myelogenous leukemia v Lymphoid antigens
- Phytohemagglutinin (= PHA) multiple lymphocyte transformation tests in healthy individuals (Transient episodes of decreased lymphocyte blastogenesis) 148
- Phytohemagglutinin blastic transformation of blood lymphocytes, v Red cell aplasia (adult, pure)
- Plasma cells of bone marrow v Cytoplasmic immunoglobulins
- Plasma folate binder comparison between the unsaturated plasma folate binder and *in vivo* labelled folate binder 296
- Plasma iron level Influence of food iron absorption on the plasma iron level in idiopathic hemochromatosis 63
- Plasma prothrombin antecedent factor (= PTA = Factor XII), v Coagulation tests
- Plasminogen activator a new method for

- the detection of the plasminogen activator content of vein walls 43
- Plasmacytosis in bone marrow aspirate, Bence Jones proteinuria
- Platelet aggregation in diabetic retinopathy 53
- Platelet count peripheral platelet count in response to salbutamol before and after adrenergic beta-receptor blockade 310
- Platelets counts in children with iron deficiency anemia 85
- Platelet membrane, human, amino groups 304
- Polycarbonate membrane filtration studies on red cell flexibility in spherocytosis, using polycarbonate membrane filtration method 329
- Polyclonal plasma cells of bone marrow Cytoplasmic haemoglobinoflavin
- Polyploidization megakaryocyte polyploidization in MAY-HESOLD anomaly 45
- Polymaturated fatty acids, Serum Epld
- Porphyria and iron metabolism, aberrations. Iron excess 369 (B)
- Porphyria, Protoporphyrin
- Practical haematology (5th ed.) 366 (B)
- Prakaidemia, Refractory anemia
- Prognosis of myelofibrosis, Myelofibrosis
- Prognostic factors in multiple myeloma (A retrospective study using conventional statistical methods and computer program) 257
- Progress, Hämorrhagische Diathesen (B)
- Propafenolol, Platelet count
- Protein, binding; folic acid binding protein in chronic granulocytic leukaemia the effect of methotrexate 341
- Protein (cytoplasmic surface of the erythrocyte membrane), Spectrin
- Proteinuria "idiopathic" Bence Jones proteinuria 269
- Protoporphyrin and haemin, binding to human spectrin 321
- PTA (= Plasma prothrombin antecedent factor = Factor XI), Coagulation tests
- Pure red cell aplasia in the adult, v Red cell aplasia (adult, pure)
- Pyruvate kinase electrophoresis relationship of density distribution and pyruvate kinase electrophoretic pattern of erythrocytes in sickle cell diseases and other disorders 201
- Rabbit erythrocyte acetylcholinesterase activity changes in the early stages of anemic and hypobaric hypoxia or after CoCl_2 treatment in rabbits 21
- Radio-carbon, v Folate binder
- Radio-chromat, v Platelet count
- Radio-iodine, v Iron uptake, Plasminogen activator
- Radio-iron, Isotopic iron absorption, Iron uptake, Plasminogen activator
- Receptor Beta-receptor blockade
- Red cell aplasia (adult, pure) paraprotoemoid variety of pure red cell aplasia (immunological studies in 1 patient) 227
- Red cell density distribution, v Erythrocytes, density distribution
- Red cell flexibility in spherocytosis, studies, using polycarbonate membrane filtration method 329
- Red cells, Erythrocyte()
- Red cells, membrane transport 366 (B)
- Red cells of mouse, Mouse red cell
- Refractory anemia clinical contribution to the knowledge of hemopoietic dysplasias long-term follow-up of 13 patients with refractory anemia 10
- Regulation of haemoglobin synthesis 127 (B)
- Remission, Megakaryocytic leukaemia
- Reticulocytes, v Myelofibrosis
- Rethkallies und lymphocytines System I. (Handb. d. Inneren Medizin, Bd. 2, Teil B) 126 (B)
- Retinopathy diabetic, platelet aggregation 53
- RETICULO-GLIOMATOSIS disease, Beta-thalassaemia

- Rosettes, v Mouse red cell, Red cell aplasia (adult, pure)
- Salbutamol: peripheral platelet count in response to salbutamol before and after adrenergic beta-receptor blockade 310
- Saudi Arabs* v Alpha-thalassaemia
- Screening coagulation tests and clotting factors in homozygous β -thalassaemia 358
- Sedimentation rate of erythrocytes, v Myelofibrosis
- Serum lactic dehydrogenase, v Myelofibrosis
- Serum lipid pattern in β -thalassaemia 100
- Serum M component, v Red cell aplasia (adult, pure)
- Serum parameters: differentiation between benign and malignant monoclonal gammopathies by discriminant analysis on serum and bone marrow parameters 129
- Serum uric acid, v Myelofibrosis
- Sickle cell diseases: relationship of density distribution and pyruvate kinase electrophoretic pattern of erythrocytes in sickle cell diseases and other disorders 201
- Sickle cell hemoglobin: blood group phenotypes and the origin of sickle cell hemoglobin in *Sicklans* 350
- Sicklans*: blood group phenotypes and the origin of sickle cell hemoglobin in *Sicklans* 350
- Sicily* β -thalassaemia in *Sicily* (Hematological and biosynthetic studies) 193
- Societies, v Hämatalogie (Tagungen)
- Souris, v Mouse
- Spectrin, human: binding of protoporphyrin and haem to human spectrin 321
- Spherocytosis: studies on red cell flexibility in spherocytosis, using a polycarbonate membrane filtration method 329
- Spherocytosis, hereditary v Erythrocytes, density distribution
- Splenic platelet pool v Platelet count
- Splenomegaly v Myelofibrosis
- Statistical methods, conventional: prognostic factors in multiple myeloma (A retrospective study using conventional statistical methods and a computer program) 257
- Statistische Auswertung, v Allogeneic bone marrow transplantation, Alpha-chain, Beta-thalassaemia, Coagulation tests, Colony formation, Cytoplasmic immunoglobulins, Discriminant analysis on serum and bone marrow parameters, Granulocyte reserve, Hypoxia, Inorganic iron absorption, Iron deficiency anemia, Iron and zinc absorption Leukaemia, lymphocytic, chronic (in Israel), Lymphocyte transformation tests, Lymphoid antigens, MAY HIGGINS anomaly Myelofibrosis, Plasma iron level, Platelet aggregation, Red cell flexibility Refractory anemia, Serum lipid, Statistical methods
- Stem cells, v Lympho-myeloid complex
- Surface of platelets, v Platelet membrane
- Synthesis of haemoglobin, regulation 127 (B)
- Tagungen, v Hämatalogie
- T (B) lymphocytes, v Red cell aplasia (adult, pure)
- T (B) rosetting lymphocytes, v Mouse red cell
- Thalassaemia, α -thalassaemia: interaction between iron deficiency and α -thalassaemia: the *in vitro* effect of haemin on α -chain synthesis 1
- Thalassaemia (β -thalassaemia): screening coagulation tests and clotting factors in homozygous β -thalassaemia 358
- serum lipid pattern in β -thalassaemia 100
- β -thalassaemia in *Sicily* (Hematological and biosynthetic studies) 193
- Therapy (excellent with Busulfan), v Mega karyocytic leukaemia
- Therapy-linked neoplasia, v Non-Hodgkin's lymphoma
- Thrombin, chemistry and biology 368 (B)
- Thrombocytes, v Platelet(s)
- Thrombosis and urokinase 126 (B)

- Thymidine (H^3 -thymidine), Lymphocyte transformation tests
- Tocopherols, v Serum lipid
- Transcobalamins, Paraproteinaemia
- Transferrin-bound iron, functional heterogeneity (Iron uptake by cell suspensions from bone marrow and liver and by cell cultures of fibroblasts and lymphoblasts) 210
- Transfusion Leitfaden des Transfusionswesens (Schriftreihe d. Akademie f. Ärztliche Fortbildung d. DDR (M. 45) 367 (B)
- Transplantation of bone marrow methotrexate and cyclosporin factor after histoincompatible allogeneic bone marrow transplants in dog 233
- Transport, membrane transport in red cells 366 (B)
- Trends in haematology 63 (B)
- Trisium, v 3H
- Tumours, growth kinetics 369 (B)
- Turkish children decreased iron and zinc absorption in Turkish children with iron deficiency and geophagia 76
- Uric acid (serum), v Myelofibrosis
- Urokinase and thrombosis 126 (B)
- Varia 128
- Vein walls new method for the detection of the plasminogen activator contents of vein walls 43
- Vergleichende Hämatologie, v Haematology comparative (B)
- Vinblastine, v Red cell sensitivity
- WALDENSTROM's macroglobulinaemia, v Monoclonal gammopathies
- Washed cells, v Mouse red cell
- White blood count, v Mouse red cell
- Whole-body counter inorganic iron absorption in subjects with iron deficiency anaemia, achylia gastrica and alcoholic cirrhosis using whole body counter 182
- Women, v Methotrexate (intrathecal)
- Zellkulturen, v Cell cultures
- Zinc and iron absorption, decreased, in Turkish children with iron deficiency and geophagia 76
- Zyto Cyto

Index autorum ad Vol. 60

(B) = Book reviews Buchbesprechungen Livres nouveaux

- Alberti, M. 358
 Ambrosetti, A. 341
 Ambrosini, M. V. 21
 Arcasoy, A. 76

 Babacan, E. 76
 Baccarini, M. 10
 Bacigalupo, A. 280
 Bambara, L. M. 341
 Barandun, S. 129 137
 Barr, R. D. 29
 Bartal, A. 148, 161
 Beaven, G. H. 321
 Beck, E. A. 366 (B)
 Benbassat, J. 257
 Benjamin, D. 117
 Benjamin, J. 316
 Bentwich, Z. 161
 Bogacka-Zatoraka, H. 122
 Braunsteiner, H. 127 (B)
 Bruch, C. 172
 Bucher, U. 63 (B)
 Bull, M. I. 233
 Burrall, P. 358

 Cambiagli, G. 291
 Caocci, L. 358
 Caramellino, L. 56
 Carella, M. 280
 Cavalli, F. 250
 Cavdar, A. O. 76

 Celada, A. 182
 Cohen, Y. 148
 Cook, J. D. 63
 Corda, R. 358
 Corrado, G. 100
 Corrocher, R. 341
 Creter, D. 53

 Damasio, E. E. 280
 Dell'Agata, M. 21
 De Sandre, G. 341
 Deubelbeiss, K. A. 126 (B),
 250
 El Gregorio, L. 193
 Dintzman, M. 117
 Dolci, C. 227
 Donath, A. 18.
 Dörmex, S. 85
 Douer, D. 117
 Droucz, W. 122
 Duckert, F. 126 (B)
 Dvllansky, A. 316

 El-Hazmi, M. A. F. 1
 Evans, C. A. 36

 Fehr, J. 62 (B)
 Finch, C. A. 63
 Fischer, A. 193
 Fiedner, T. M. 172
 Fredén, K. 310
 Furlan, M. 368 (B)

 Ganzoni, A. † 369 (B)
 Giardini, O. 100
 Giovannini, E. 21
 Gonzalez, J. 269
 Grassi, G. 21
 Gratwohl, A. A. 233
 Gratzner, W. B. 321
 Graw, R. G. Jr. 233

 Haasz, R. 148
 Herbst, E. W. 172
 Herreros, V. 182
 Heyns, A. du P. 296
 Hijašovec, G. 85
 Huser, H. J. 137

 Izak, G. 161

 Jedrzejewski, R. 12.
 Johnson, R. H. 243
 Johnson, R. 379

 Kahn, Y. 59
 Kaplan, H. 316
 Knutzen, T. 233
 Kottenhagen, M. J. 210
 Kovács, P. 172
 Kuttli, J. 310

 Lehmann, H. 1
 Lefjose, B. 210
 Levine, J. 269
 Lewis, A. 56

- Lint, M. T. van 280
 Loefiger E. A. 365 (B)
 Lopes-Virella, M. F. 269
 Ludin, J. 64 (B)
 Lundsgaard-Hansen, P.
 64 (B)

 Maggiori, G. 100
 Mazzerolo, O. 100
 Mauny N. 161
 Mansfield, A. O. 243
 Marmont, A. M. 90, 280,
 368 (B)
 Marti, H. R. 367 (B)
 Martino, F. 100
 Matzner Y. 257
 Maurer W. 129
 Mayer M. 45
 Mitzewski, B. 122
 Miskori, T. 148
 Miescher, P. A. 128 (B)
 Mulder M. S. 65
 Morell, A. 129 137
 Mulder H. J. M. 210
 Mulder N. H. 108
 Murgia, F. 100
 Muzumeci, S. 193, 350

 Najean, Y. 127 (B), 369 (B)
 Nicotas, A. 304
 Nien-eg, H. O. 108
 Noordeloos, P. J. 210
 Nortas, L. 233

 Oda, E. 201
 Oda, S. 201
 Oelofse, R. 296
 Ogrwa, M. 269
 Olsson, L.-B. 310
 Oosthuizen, M. 296

 Pachor M. L. 341
 Palma, S. 291
 Palestro, G. 227
 Paolino, F. 56
 Paolino, W. 56
 Pavlotzky F. 53
 Pegrum, G. D. 36
 Peschle, C. 227
 Puggio, G. 280
 Pick, A. I. 117
 Pinkhas, J. 117
 Pizzarelli, G. 193
 Pollack, A. 257
 Porembófska, H. 122
 Principato, G. B. 21

 Quisner W. 45

 Rachmifewitz, E. A. 350
 Raffo, M. C. 280
 Raenen, O. R. van 296
 Renegotti, L. 227
 Ratlef, F. P. 296
 Ricci, P. 10
 Rhao, M. 280
 Robinson, E. 148
 Rojer R. A. 108
 Rudolf H. 182
 Russo, A. 350
 Russo, G. 193
 Rysael, H. J. 280

 Sandler S. G. 350
 Santini, G. 280
 Santucci, M. A. 10
 Savir, H. III
 Schaefer J. 45
 Schatzmann, H. J. 366 (B)
 Schöffelb, G. 193 350
 Seidl, S. 367 (B)
 Skvartil, F. 129 137
 Sonntag, R. W. 250
 Speck, B. 126 (B)
 Spengler G. 137
 Sperling, H. 45
 Stoltz, J. F. 304
 Stöglitz, G. III
 Stuetz, K. 85

 Temska, K. R. 201
 Tora, S. 10

 Verboef, N. J. 210
 Vlito, L. 310
 Virella, G. 269
 Vorobiof, D. A. 316
 Vuopio, P. 329

 Watt, J. 29
 Weiss, S. 99
 Wrodska, J. 122

 Zaccaria, A. 10
 Zer M. 117
 Zinnermann, A. 250

Index autorum ad Vol. 60

(B) = Book reviews / Buchbesprechungen / Livres nouveaux

- Alberti M. 358
 Ambrosetti A. 341
 Ambrosini M V 21
 Arcasoy A 76

 Babacan, E. 76
 Baccarini, M. 10
 Bacigalupo, A. 280
 Bambara, L. M 341
 Barandun S. 129 137
 Barr R. D 29
 Bartal, A. 148 161
 Beaven, G H. 321
 Beck, E. A. 366 (B)
 Benbasat, J 257
 Benjamin, D 117
 Benjamin, J 316
 Bentwich, Z. 161
 Bogacka-Zatoraka, H 122
 Braunsteiner H. 177 (B)
 Bruch C. 172
 Bucher U 63 (B)
 Bull M I. 233
 Burrai P 358

 Camblaghi G 291
 Caocci L. 358
 Caramellino L. 56
 Carella, M 280
 Cavalli, F 250
 Cavdar A. O 76

 Celada, A. 18
 Cohen, Y 148
 Cook, J D 63
 Corda, R. 358
 Corrado, G 100
 Corrocher R. 341
 Creter D 53

 Damasio E. E. 280
 Dell Agata, M 21
 De Sandre, G 341
 Denbelsa, K. A. 176 (B),
 250
 Di Gregorio L. 193
 Dintzman, M. 117
 Dolci C. 227
 Donath, A. 182
 Dönmez, S. 85
 Douer D 117
 Droszcz, W 122
 Duckert, F 126 (B)
 Dvilansky A. 316

 El-Hazmi, M. A. F 1
 Evans, C. A. 36

 Fehr J 62 (B)
 Finch C. A. 63
 Fischer A. 193
 Fliedner T M 172
 Fredén, K. 310
 Furlan, M 368 (B)

 Gannoni A. † 369 (B)
 Giardini O 100
 Giovannini, E. 21
 Gonzalez, J 269
 Grassi, G 21
 Gratwohl, A. A. 233
 Gratzner W B. 321
 Graw R. G Jr 233

 Haasz, R. 148
 Herbst, E. W 172
 Herreros, V 182
 Heyna, A. du P 296
 Hicobomez, G 85
 Huser H J 137

 Izak, G 161

 Jedrzejewski, R. 122
 Johnson, R. H 243
 Johnson, R. 379

 Kahn, Y 59
 Kaplan, H. 316
 Kmitzen, T 233
 Kottenhagen, M. J 210
 Kovács, P 172
 Kutil, J 310

 Lehmann, H 1
 Leijne, B. 210
 Levine, J 269
 Lewis, A. 56

